The characterisation of polymicrobial bacterial communities in the lower respiratory tract of individuals with chronic pulmonary disease

Paul J. Purcell

PhD

2013

The characterisation of polymicrobial bacterial communities in the lower respiratory tract of individuals with chronic pulmonary disease

Paul J. Purcell

A thesis submitted in partial fulfilment of the requirements of the University of Northumbria at Newcastle for the degree of Doctor of Philosophy

Research undertaken in the School of Life Sciences and in collaboration with the Freeman Hospital, Newcastle upon Tyne

January 2013

Publications relating to thesis

Publications

Purcell, P.J., Jary, H., Nicholson, A. Perry, J.D., Lanyon, C., Smith, D., Cummings, S.P., De-Soyza, A. (2012). Polymicrobial airway bacterial communities in adult non-CF bronchiectasis patients (in preparation for submission).

Purcell, P.J., Cummings, S.P., Jary, H., De-Soyza, A., & Perry, J.D. (2012). Changes in the lower airway bacterial communities from adult non-CF bronchiectasis populations are significantly associated with exacerbations and the presence of *Haemophilus influenzae*. *European Respiratory Journal* **40**: Supplement 56: 450s.

Purcell, P.J., Nelson, A., Fisher, A., Perry, J.D., De-Soyza, A., & Cummings, S.P. (2011). Molecular fingerprinting and metagenomic analysis reveals a polymicrobial element in patients with chronic obstructive pulmonary disease. *Thorax* **66**: Supplement 4: A11-A12.

Cummings, S.P., Nelson, A., Purcell, P.J., De-Soyza, A., Bourke, S.J., & Perry, J.D. (2010). A comparative study of polymicrobial diversity in CF and non-CF bronchiectasis. *Thorax* **65**: Supplement 4: A13-A14.

Oral presentations

Purcell, P.J., Nelson, A., Fisher, A., Perry, J.D., De-Soyza, A., & Cummings, S.P. (2011). Molecular fingerprinting and metagenomic analysis reveals a polymicrobial element in patients with chronic obstructive pulmonary disease. Presented at the British Thoracic Society Winter Meeting, London, 7th December 2011.

Purcell, P.J., Nelson, A., & Cummings, S.P. (2009). Comparison of DNA polymerase enzymes in DGGE studies of bacterial communities in soil. Presented at the 15th Molecular Microbial Ecology Group Meeting, Aberdeen University, 30th July 2009.

Poster presentations

Purcell, P.J., Cummings, S.P., Jary, H., De-Soyza, A., & Perry, J.D. (2012). Changes in the lower airway bacterial communities from adult non-CF bronchiectasis populations are significantly associated with exacerbations and the presence of *Haemophilus influenzae*. Presented at the European Respiratory Society Annual Congress Conference, Austria, Vienna, 3rd September 2012.

Purcell, P.J., De-Soyza, A., Jary, H., Perry, J.D., Cummings, S.P. (2011). PCR-DGGE mediated studies of non-CF bronchiectasis individuals reveals a polymicrobial community. Presented at the Society for General Microbiology Spring Conference, Harrogate International Centre, 13th April 2011.

Abstract

Microbial diversity encompasses the whole of the Earth's biosphere and is incredibly vast. The microbial diversity of three disparate micro-environments using two culture-independent techniques (denaturing gradient gel electrophoresis (DGGE) and 454-pyrosequencing) were revealed. Five commercially available DNA polymerase (pol) enzymes were assessed in determining the bacterial community generated in sandy soil. The V3 region of the 16*S* rRNA gene was targeted for amplification by polymerase chain reaction (PCR). Using a PCR-DGGE approach, different DNA pols exhibited differences in the DGGE profiles produced. Both high-fidelity DNA pols *Ex Taq*™ Hot Start (HS) and Platinum® *Pfx* detected greater microbial diversity present within sandy soil than the other DNA polymerase enzymes.

We employed *Ex Taq*™ HS to characterise the microbial communities present in two chronic respiratory tract diseases, non-cystic fibrosis bronchiectasis (nCFBR) and chronic obstructive pulmonary disease (COPD). Seventy individuals expectorated sputum, and using 16*S* and 28*S* rRNA PCR-DGGE polymicrobial communities were revealed. From the 70 patients investigated, 20 presented with symptoms consistent with an exacerbation, the remainder being clinically stable. Demographic and culture data were used in constrained ordination analyses to identify any significant associations between these data and changes in the sputum microbiota. The data presented indicates that bacterial lung communities in adult nCFBR patients have distinct differences between exacerbating and clinically stable episodes. Persistent colonisation by *Pseudomonas aeruginosa* is significantly associated with reduced lung function, and is negatively correlated with *Haemophilus influenza*e carriage. Bacterial communities seem to be predominantly assembled by stochastic processes. Fungal taxa present were scarce.

Stable COPD populations have been previously investigated using culture-dependent techniques. Eleven clinically stable COPD patients had a bronchoalveolar lavage (BAL) fluid taken from the right lower lobe. Both 16*S* and 28*S* rRNA PCR-DGGE was performed on all clinical samples from extracted DNA. Co-migration of bands was then compared to a 16*S* and 28*S* standard ladder consisting of pure cultivars. Additionally, execution of 454-pyrosequencing and interrogation of the V3-V5 region of 16*S* rRNA genes resulted in 1799 unique OTUs being identified. Dominant bacterial genera identified were *Streptococcus*, *Arthrobacter*, and *Staphylococcus* respectively. Bacterial taxa identified were then subjected to multivariate statistical analysis to identify relationships between the microbial communities and patient phenotypes. Metagenomic analysis demonstrated that heterogeneous bacterial populations exist in all eleven individuals. This preliminary study shows that the lungs of COPD sufferers are colonised with multiple species of bacteria and demonstrate that a complex microbial community is present. Furthermore, bacterial phylotypes resolved to class-level indicated three potential drivers of community structure within the COPD lung microbiome: lung function, moderate and severe COPD progression, and smoking status in cohort. The identification of a greater number of bacterial taxa was also apparent in culture-negative patients using both PCR-DGGE and 454-pyrosequencing approaches.

List of tables

List of figures

For my parents who will always believe in me.

And for Josephine Purcell, Peter Purcell,

Glen Purcell, Keith Purcell, and Lorraine Purcell, RIP.

"The Earth is a microbial planet, on which macro-organisms are recent additions — highly interesting and extremely complex in ways that most microbes aren't, but in the final analysis relatively unimportant in a global context" (Prof. Mark Wheelis in a personal communication to Prof. Carl Woese, 1998).

Acknowledgements

I remember when I first enrolled at Northumbria University way back to embark on a road of reading for the BSc award. Even back then, I have always wanted to fulfil my ambition of submitting for a Ph.D. The road leading to this realisation could not have been possible without certain influences, events, and the many people that I have been introduced to in my life. The following individuals below have been instrumental to me in achieving this goal.

I would personally like to thank Prof. Stephen Cummings for giving me this unique opportunity in addition to his guidance, forethought, encouragement, and patience in all aspects in the body of work contained herein.

Additional thanks to Prof. John Perry and Audrey Perry for extra support in the clinical microbiology aspects and collection of patient clinical specimens, without which, this project would not have been achievable. I would like to show my gratitude to you both for allowing me to carry out the required genomic DNA extractions and the use of the facilities at the Freeman Hospital, Microbiology Department.

Thanks also to Dr. Anthony De-Soyza for his invaluable knowledge, thoughtful suggestions and discussions, especially in regards to the more clinical aspects of the research project; in particular, patient recruitment and selection. A special mention must be given to Dr. Hannah Jary for organising and complying all of the patient phenotypic data in the non-CF bronchiectasis cohort. Additional thanks also to Dr. Syba S. Sunny for providing extra clinical information regarding the cross-sectional non-CF bronchiectasis patient cohort.

I owe a great deal of gratitude to my immediate research colleagues and friends in the Cummings group: Jesmine Lim for thoughtful discussions; Dr. Andrew Nelson for his invaluable advice and direction in relation to the technical aspects of the project; Dr. Caroline Orr for her statistical intuition and guidance; and Chris Stewart for his tuition in the metagenomic aspects of this project. I would like to thank you all for your support and kindness.

I would like to collectively thank colleagues past and present who I had the pleasure of working alongside with not only in Lab A321 but also within the School of Life Sciences: Paul Agnew, Linda Barlow, Vivien Brindley, Dr. Alistair Brown, Dr. Lynn Dover, Susan Carlile Harry Elliott, David Harrison, Beth Gibson, Dr. Alan Jones, Dr. Amanda Jones, Dr. Clare Lanyon, Dr. Anna Lindsay, Suzanne Lonsdale, Dr. Peter Maskrey, Fiona McKie-Bell, Dr. Kay Paget, Deborah Pettitt, Dr. Richard Ranson, Dr. Steve Reed, Dr. Bryan Singer, Dr. Darren Smith, Derek Stobo, David Thomas, Dr. Hannah Walden, Karen Walker, Jennifer Wright, and Robert Young. A special thanks to the following colleagues, as without their encouragement as an undergraduate and I would not have pursued this goal: Dr. Chris Baldwin, Prof. Gary Black, Dr. Geoff Bosson, Dr. Mark Daniels, Dr. David Holmes, and Prof. Iain Sutcliffe.

I would also like to give thanks to the many Ph.D. researchers that I have met and worked with on this journey (you know who you are and you are too numerous to mention!). Additional thanks to Lee Barrett for the endless debates on politics, film, music and assorted subject matters! I also offer my thanks to my close friends and colleagues at M&S.

Finally, I owe a great deal of thanks to four very special people in my life: mum, dad, Simon and Natalie (who has at times endured this Ph.D. with me and kept me going when the going was very tough). Thank you all for your tireless support, patience and love in helping me achieve this goal.

Declaration:

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others. The work was carried out in collaboration with the Microbiology Department, Freeman Hospital, Newcastle upon Tyne.

Name:

Signature:

Date:

List of abbreviations

A:	adenine
AIDS:	acquired immunodeficiency syndrome
amoA:	gene encoding ammonium oxygenase enzyme(s)
AB:	AmpliTaq® DNA polymerase (Applied Biosystems™)
ANOVA:	analysis of variance
APS:	ammonium persulphate
bTEFAP:	bacterial tag-encoded FLX-Titanium amplicon pyrosequencing
BAL:	bronchoalveolar lavage
BALF:	bronchoalveolar lavage fluid
BCC:	*Burkholderia cepacia* complex
bp:	base pairs
BPB:	bromophenol blue
BS:	bronchial secretion
BSA:	bovine serum albumin
C:	cytosine
cAMP:	cyclic-adenosine monophosphate
CCA:	canonical correspondence analysis
cDNA:	complementary deoxyribonucleic acid
CF:	cystic fibrosis
CFTR:	gene encoding the CF transmembrane conductance regulator protein

cm: centimetre(s)

CO_2: carbon dioxide

COPD: chronic obstructive pulmonary disease

CS#X: COPD subject where X denotes patient number enrolled in study

DCA: detrended correspondence analysis

DCs: dendritic cells

DGGE: denaturing gradient gel electrophoresis

dH_2O: distilled water

DHLPC: denaturing high-performance liquid chromatography

DPI: dots per inch

DNA: deoxyribonucleic acid

dNTPs: deoxyribonucleoside triphosphates

dsDNA: double-stranded deoxyribonucleic acid

dsRNA: double-stranded ribonucleic acid

EDTA: ethylenediamine tetraacetic acid

ET: *Ex Taq*™ Hot Start DNA polymerase (TAKARA BIO INC.)

EPS: extracellular polymeric substances

FEV_1: forced expiratory volume in one second

FEV_1%: forced expiratory volume in one second predicted

FVC: forced vital capacity

g: × acceleration under gravity

g: gram(s)

G: guanine

gDNA: genomic deoxyribonucleic acid

GOLD: Global Initiative for Chronic Obstructive Lung Disease

GT: GoTaq® Hot Start DNA polymerase (Promega)

h: hour(s)

H: hydrogen

H': Shannon diversity index

HCl: hydrochloric acid

HIV: human immunodeficiency virus

HMP: Human Microbiome Project

HPMV: human metapneumovirus

HCPC: Health & Care Professions Council

HRCT: high-resolution computed tomography

HS: hot start

IgA: immunoglobulin A

IL-1: interleukin 1

J': species evenness

L: litre

LB: Luria-Bertani

LMG: Laboratory of Microbiology, University of Ghent

ln: natural logarithm

LPS: lipopolysaccharide

LRR: leucine-rich-repeat

LRT: lower respiratory tract

LTA: lipoteichoic acid

M molar

m: metre(s)

mA: milliamp(s)

mg: milligram(s)

Mg^{2+}: magnesium cation

MΩ mega ohm(s)

$MgCl_2$: magnesium chloride

$MgSO_4$: magnesium sulphate

min: minute(s)

MM: master-mix

Mn^{2+}: manganese cation

µ: micro

n: nano

NAD: nicotinamide dinucleotide

NaCl: sodium chloride

NaOH: sodium hydroxide

NBD1: nucleotide binding domain 1

NCDs: non-communicable diseases

nCFBR: non-cystic fibrosis bronchiectasis

NCTC: National Collection of Typed Cultures

NGS: next-generation sequencing

nifH: gene encoding nitrogenase enzyme(s)

NIH: National Institutes of Health

NKCs: natural killer cells

nt: nucleotide(s)

NTHI: non-typeable *Haemophilus influenzae*

NTM: non-tuberculosis mycobacteria

O_2: diatomic oxygen

OTU: operational taxonomic unit

PAMPs: pathogen associated molecular pattern(s)

PC: principal component

PCA: principal components analysis

PCoA: principal co-ordinates analysis

PCR: polymerase chain reaction

Pg: petagram(s)

Pfx: Platinum® *Pfx* DNA polymerase (Invitrogen™)

pol: polymerase(s)

PPi: pyrophosphate

PPMs: potentially pathogenic micro-organisms

PRRs: pattern-recognition receptors

QS: quorum sensing

R: species richness

RDA: redundancy analysis

rDNA: ribosomal deoxyribonucleic acid

Rf: relative front

RLL: right-lower lobe

RNA: ribonucleic acid

rpm: revolutions per minute

rpoB: gene encoding for RNA polymerase β subunit

rRNA: ribosomal ribonucleic acid

rrn: operon encoding 16*S* rRNA, 23*S* rRNA, and 5*S* rRNA genes

rrs: gene encoding 16*S* rRNA

RSV: respiratory syncytial virus

RTI: respiratory tract infection

RTL: Research and Testing Laboratories, LLC.

s: second(s)

S: Svedberg unit(s)

SD: standard deviation

SDS: sodium dodecyl sulphate

SL: standard ladder(s)

SOP: standard operating procedure

spp.: species

SSCP: single strand conformation polymorphism

ssDNA: single-stranded deoxyribonucleic acid

SSU: small subunit

SSXR: soil sample X (denotes sample number) replicates

T: thymine

TAE: tris-acetate-EDTA

Taq: New England Biolabs® Inc. *Taq* DNA polymerase

TEMED: *N*,*N*,*N*′,*N*′-tetramethylethylenediamine

TGGE: temperature gradient gel electrophoresis

TIFF: tagged image file format

TLRs: Toll-like receptors

ToL: tree of life

T-RFLP: terminal-restriction fragment length polymorphism

URT: upper respiratory tract

UV: ultra-violet

V: volt(s)

WHO: World Health Organization

1°: primary

3′: 3 prime

5′: 5 prime

Chapter One: General introduction

1.1 A global view of micro-organism biodiversity

The biosphere of Earth is absolutely dependent on micro-organisms since they are required to sustain almost every form of life on the planet and are also key drivers in almost all geochemical cycles (Madigan et al., 2009). Since the landmark paper published by Woese and Fox in 1977, followed by subsequent developments in molecular biology in the 1980s, microbial diversity on Earth is recognised as being greater than microbiologists had ever envisioned. Over evolutionary time, diversity has led to the successful proliferation and colonisation of micro-organisms across an extensive range of habitats. Environments such as soil, the ocean, the subsurface, and indeed macro-organisms including humans, harbour an extraordinary number of prokaryotes (Whitman et al., 1998). In addition, the many prokaryotic phyla have species capable of colonising a multitude of environments inhospitable to other forms of life for example: hot springs situated in Yellowstone Park (Hugenholtz et al., 1998b), the high temperatures and extremely low pH niche of the thermoacidicphilic euryarchaeon *Picrophilus torridus* (Futterer et al., 2004), the deep sea (DeLong et al., 1994), hydrothermal vents (Huber et al., 2000), the sea ice of the Antarctic (Thomas and Dieckmann, 2002), and even in the permanent ice both in Lake Bonney and Lake Vostok (Christner et al., 2001, Gordon et al., 2000, Priscu et al., 1999).

Estimates of the total number of prokaryotes on Earth are in the order of 4-6 $\times$ 10^{30} cells with a biomass of 350-550 Pg of carbon (1 Pg = 10^{15} g) (Whitman et al., 1998). Owing to the inconceivable numbers of bacterial cells present in our biosphere, their rapid growth and ability to demonstrate physiological and genetic adaptability this group of organisms has incredible genetic diversity (Hindré et al., 2012). Putting these numbers into perspective is indeed a daunting task, it has been estimated that one ton of soil contains at least 4 $\times$ 10^{6} different taxa (Curtis et al., 2002). Quoting Pace, *et al*., "we are only scratching the surface of a vast reservoir of microbial diversity" (Pace et al., 2012).

1.2 The origins of modern molecular microbiology

A sobering aspect to consider in terms of the many species of bacteria that inhabit the Earth is that estimates of only 1 % of all microbial species have been identified and characterised by classical culture-based techniques (Amman et al., 1995, Staley and Konopka, 1985). Historically, before the advent of molecular sequence-based phylogenetics much of microbial life (and its genetic diversity) was alluded to and previous hypotheses regarding the kingdoms of life constituted five: animals, plants, fungi, protists, and monera (protozoa and bacteria respectively) (Whittaker, 1969). This model was adopted primarily on the previous knowledge of the macro-organisms around us within the biosphere, and rather ignorantly, bacteria themselves were thought to be unimportant; the term "prokaryote" itself was defined rather negatively in comparison to their eukaryotic counterparts in the 1960s (Stainer and van Niel, 1962). The advances in molecular biology in the next decade made possible the inference of phylogenetics across all the kingdoms of life by comparison of ribosomal ribonucleic acid sequences. In addition to the seminal work carried out by Pace and collaborators using these techniques enabled a reconstruction of the history of life and proposed a new model consisting of the now-recognised three primary lines of evolutionary descent as opposed to the initial standard two in the universal tree of life (Woese, 1987, Woese and Fox, 1977, DeLong and Pace, 2001, Olsen et al., 1986). These are now formally designated "urkingdoms" or "domains" represented by the Eucarya (eukaryotes), Bacteria (initially termed the eubacteria) and Archaea (initially termed archaebacteria) (Fig. 1.1) (Woese et al., 1990).

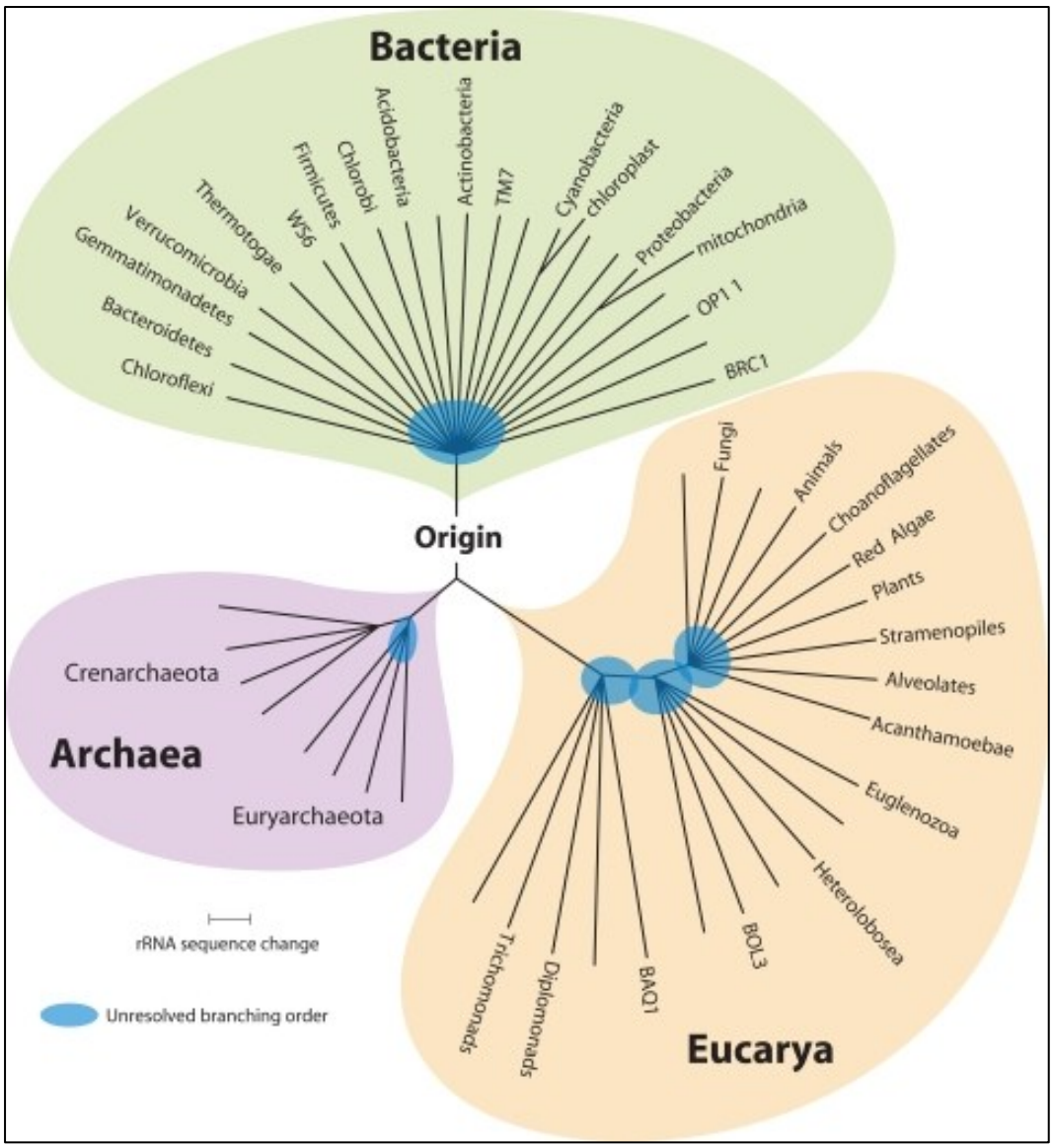


Fig. 1.1: The universal tree of life (ToL) representing the three known domains, Archaea, Bacteria, and Eucarya. These domains or kingdoms were phylogenetically reconstructed using partially or fully sequenced ribosomal ribonucleic acid sequences (rRNAs) from both the 16*S* (ubiquitous in archaeal and bacterial species) and 18*S* (exclusive to the Eucarya domain) ribosomal small subunits. Phylum levels indicated are shown by the branches in each kingdom, whereas at the bases of each phylogenetic tree there are unresolved branching orders (blue circles) (Pace, 2009).

1.3 Culture-dependent microbiology

Classical culture-based microbiological techniques pioneered by Beijerinck, Koch, Pasteur, van Leeuwenhoek, and Windogradsky maintained and developed microbiological research for centuries (Keller and Zengler, 2004). Culture-dependent microbiology is crucial if one is to characterise and gain a significant understanding of a bacterial species or micro-organism, its physiology, or access to its metabolic pathways via genes dispersed throughout its genome (Keller and Hohn, 1997, Palleroni, 1997, Yu et al., 2002). However, using traditional enrichment and cultivation techniques accepts the issue that such techniques are highly selective and biased towards the growth of specific bacterial species.

This facet of traditional culture-based microbiology is no more apparent than in the modern clinical microbiology laboratory where these techniques are used in conjunction with modern molecular-based methodologies for the routine isolation of potentially pathogenic micro-organisms (PPMs) from patient specimens (i.e., the detection of aetiological agents by providing conditions they require to grow *in vitro* (Rogers et al., 2009a)). Indeed, using selective media for effective isolation requires that the PPMs in question must grow to at least 10^5 cells in density in order for these bacterial colonies to be visualised (Keller and Zengler, 2004). One must also bear in mind that for isolation of the aetiological agent(s), the selective media employed biases in favour of fast-growing and high density forming micro-organisms that have the capacity to withstand high concentrations of nutrients and have the ability to grow in isolation (Keller and Zengler, 2004). A great number of traditional cultivation techniques fail to isolate many micro-organisms from most natural habitats, including humans. A major contributing factor of this, is that the traditional cultivation techniques employed such as selection and isolation of specific microbes utilise conditions for growth *in vitro* that are completely different to the natural environment from which many of these micro-organisms originate from (Eilers et al., 2000, Torvisk et al., 2002).

The major limiting factor in using culture-based microbiology to isolate aetiological agents is that culture-based techniques do not consider the role of polymicrobial infections or the vast numbers of microbial taxa that colonise the human host. Indeed, bacteria infecting human tissues are often part of mixed bacterial communities, in particular when mucosal barriers have been compromised (Brogden et al., 2005). Contextually, we sometimes need to consider infection as a complex microbial milieu (Rogers et al., 2009a). This is very

important if appropriate treatment regimens are to be adopted and tailored to the individual patient infected with one or more pathogenic organisms. The advent of culture-independent techniques has revealed these microbial communities within the human host in greater detail than ever before and also enables investigators to appreciate the magnitude of the task involved in characterising these communities.

1.4 Culture-independent microbiology

Culture-independent techniques have revealed a vast world of microbial diversity remaining to be discovered and placed microbes, once thought to be insignificant, firmly back into their respective positions in the universal tree of life (Pace et al., 2012). Molecular fingerprinting methods such as denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE), terminal restriction fragment length polymorphism (T-RFLP), single strand conformation polymorphism (SSCP), and denaturing high-performance liquid chromatography (DHLPC) have enabled researchers to analyse microbial communities from several ecosystems; these techniques are collectively known as microbial profiling (Nocker et al., 2007). These fingerprinting techniques have usually targeted the 16*S* rRNA gene(s) (Hugenholtz et al., 1998a, Pace et al., 1986) or sometimes functional genes encoding, for example, ammonium mono-oxygenase (*amoA*) (Horz et al., 2000, Yeager et al., 2005), nitrogenase (*nifH*) (Rosado et al., 1998, Widmer et al., 1999), and RNA polymerase B (*rpoB*) (Adékambi et al., 2008, Ormeno-Orrillo et al., 2006, Renouf et al., 2006) for the analysis of sub-populations that exhibit certain functional properties within a community (Nocker et al., 2007). Total community DNA is extracted and used as a template for PCR amplification of the 16*S* rRNA gene(s), PCR fragments are then either analysed by electrophoresis (DGGE, TGGE, SSCP, and T-RFLP) or chromatography (DHLPC) (Nocker et al., 2007). Resolution of the different bacterial species within the community is achieved as a function of DNA sequence variation within the amplified 16*S* rDNA fragments as these are usually of similar size (Hamady and Knight, 2009, Nocker et al., 2007). Finally, from the community profiles generated, one can analyse the banding patterns or clustering of specific bands using statistical techniques such as Principal Co-ordinates Analysis (PCoA) (Dollhopf et al., 2001).

More recently, the next-generation sequencing (NGS) analysers developed by Roche, Illumina®, and Applied Biosystems™ have taken sequencing to the next level via deployment of multiple platforms and different sequencing technologies paving the way for the era of metagenomics that we now live in. This 'next level' can be further defined as what is commonly known as 'deep sequencing', i.e., massively parallel sequencing — the simultaneous sequencing of several hundred thousand DNA fragments with read lengths exceeding that of 100 base pairs (bp) (McPherson, 2009). The term metagenomics is defined as "culture-independent studies of the collective set of genomes of mixed microbial communities and applies to explorations of all microbial genomes in consortia that reside in

environmental niches, in plants, or in animal hosts (Petrosino et al., 2009). Using 454-pyrosequencing as an example of a NGS platform; briefly, DNA pyrosequencing or sequencing synthesis is based on the detection of released inorganic pyrophosphate (PPi) during DNA synthesis. Visible light (i.e., photons) is generated by a cascade of enzymatic reactions and the amplitude of each light signal is proportionally represented by the incorporation of nucleotides (Fig. 1.2) (Novais and Thorstenson, 2011). In metagenomic studies using massively parallel sequencing technology, DNA pyrosequencing targets hypervariable regions within bacterial 16*S* rRNA genes amplified by PCR. By using millions of single-stranded DNA fragments from isolated genomic DNA (gDNA) as sequencing templates attached to beads in emulsion PCR, amplification occurs within each emulsion droplet. Each bead carrying millions of copies of unique DNA templates are all denatured and every bead attached to a single-stranded DNA (ssDNA) clone are deposited into tiny chambers before being exposed to another set of smaller beads carrying immobilised enzymes which are responsible for DNA pyrophosphate sequencing. Nucleotide composition of each ssDNA fragment is elucidated by the associative release of PPi and generation of photons (Margulies et al., 2005). Exploitation of 16*S* rRNA gene(s) in culture-independent studies of the microbiota of metazoan subjects and the environment has enabled microbial ecologists to not only infer the phylogenetic relationships of the bacterial communities but to also detect uncultivable species of bacteria from many taxa using modern sequencing technologies coupled with molecular fingerprinting methodologies. From the original 11 phyla described by Woese and colleagues nearly 30 years ago (Woese et al., 1985), the bacterial phyla now number 53 thanks in part to the impact of 16*S* rRNA gene sequencing from uncultivable bacterial species (Rappé and Giovannoni, 2003).

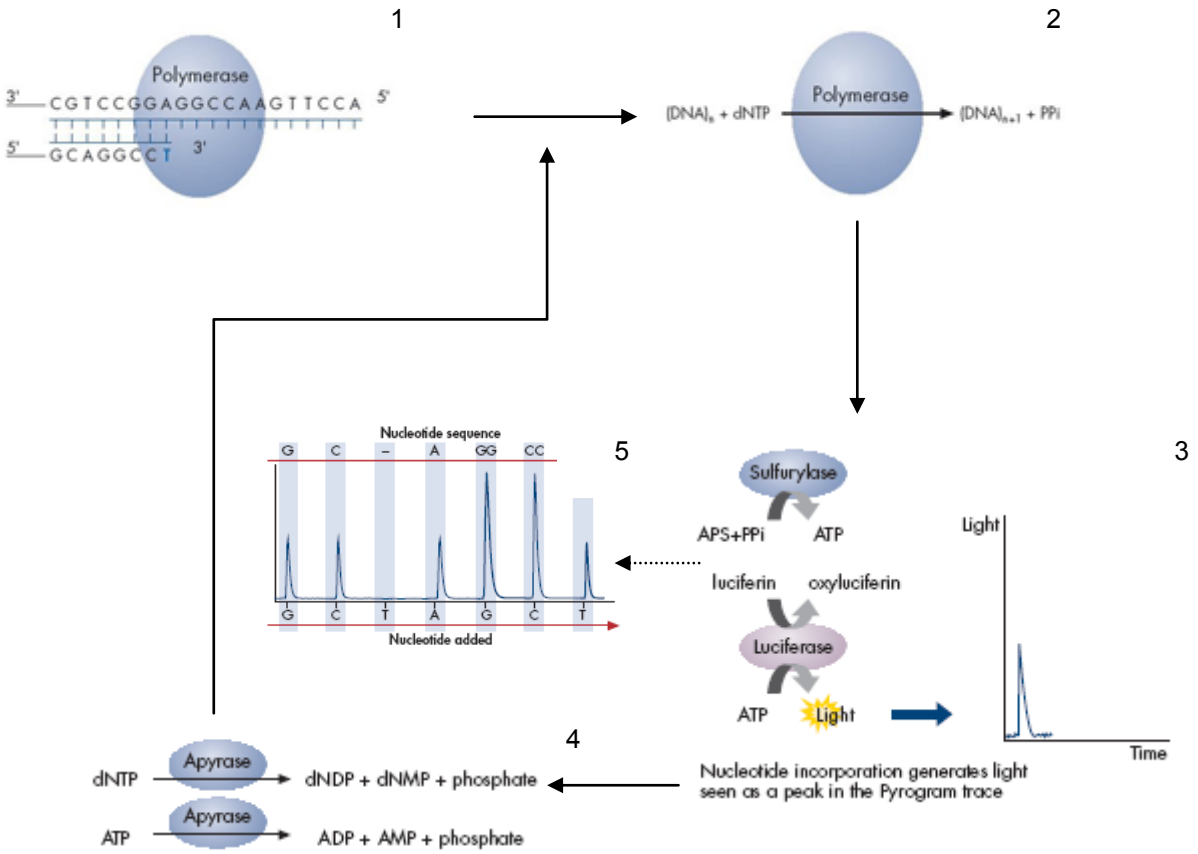


Fig. 1.2: Principals of DNA pyrosequencing. 1.) Single-stranded DNA (ssDNA) in PCR amplicon serves as template for hybridisation of a sequencing primer incubated with four enzymes, DNA polymerase, adenosine triphosphate (ATP) sulphurylase, luciferase, and apyrase in addition to the substrates adenosine 5′ phosphosulphate (APS), and luciferin. 2.) Addition of the first deoxyribonucleoside triphosphate (dNTP) into the reaction results in catalysis of this dNTP by the DNA polymerase enzyme via insertion into the DNA strand but only if it is complementary to the base in the template strand. Incorporation of these complementary bases is associated with the release of inorganic pyophosate (PPi). Subsequently, the amount of PPi released is related to the amount of incorporated nucleotides in the reaction, i.e, in equimolar quantities. 3.) Conversion of PPi to ATP is executed by the enzyme ATP sulphurylase in the presence of APS. The generation of photons proportional to the amount of ATP released from the primary conversion from PPi to ATP is from the ATP product itself driving the luciferase-mediated conversion of luciferin to oxyluciferin. Detection of photons in the luciferase-catalysed reaction is achieved by a charge coupled device chip and is visualised by a peak in the program (i.e., raw data output). The light signal produced is proportional to the number of nucleotides incorporated. 4.) The nucleotide-degrading enzyme, apyrase, continuously degrades unincorporated nucleotides and ATP. Subsequent addition of nucleotides only occurs once this degradation process is complete. 5.) The complementary DNA strand is synthesised continually by the sequential addition of dNTPs in which the nucleotide sequence data is determined by the signal peaks visualised in the program output and then annotated appropriately (Novais and Thorstenson, 2011, Qiagen). Abbreviations: ADP, adenosine diphosphate; AMP, adenosine monophosphate; dNDP, deoxyribonucleoside diphosphate; dNMP, deoxyribonucleoside monophosphate.

In particular, culture-based microbial identification to nucleic acid sequencing is now becoming more commonplace in diagnostic microbiology laboratories. In the past this approach has been infrequently adopted due to the technical expertise needed to perform DNA sequencing using Sanger technologies (Petrosino et al., 2009). Developments using Sanger-sequencing showed that 16*S* rRNA genes contain 9 hypervariable regions, V1-V9, in which ~ < 500 bp of the 16*S* rDNA coding sequence was required in order to generate genus- and species-level pathogen identification (Kolbert and Persing, 1999, Lane et al., 1985). The hypervariable V1-V9 regions (50-100 bases in size) situated within the 16*S* rRNA genes in all bacteria are interspersed between highly conserved regions (Fig. 1.3); indeed, all 16*S* rRNA vary in their nucleotide sequence composition, but the conserved regions remain as they were, unchanged, conserved invariant through time across nearly all bacterial species due to their essential functions within the bacterial cell (van de Peer et al., 1996, Jonasson et al., 2002). This key property coupled with the inherent nature of 16*S* rRNA in addition to high information content and universal distribution reveals why 16*S* rRNA is utilised for phylogenetic analysis, microbial community profiling, and more recently, metagenomic studies using NGS technology.

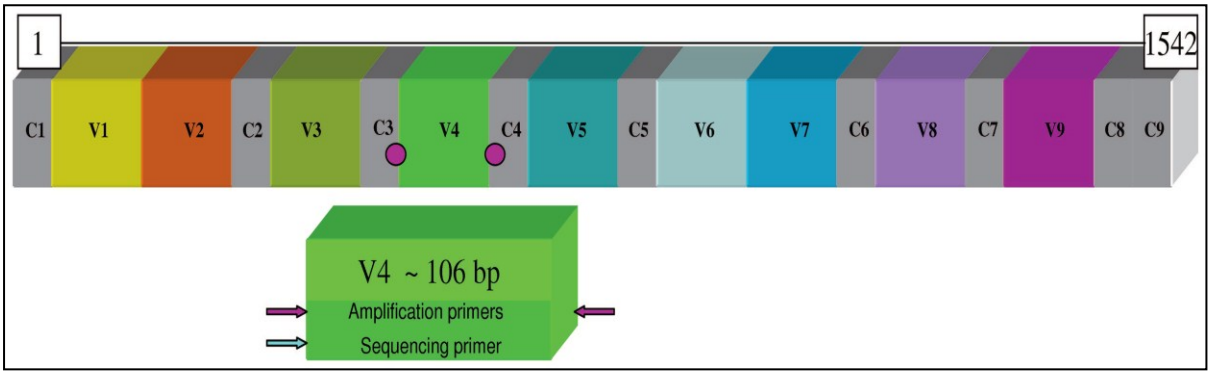


Fig. 1.3: Conserved and hypervariable regions within the 16*S* rRNA gene. The conserved regions interspersed throughout this gene are indicated in grey (C1-C9), whereas the hypervariable regions (V1-V9) are represented by different colours. Each hypervariable region ranges from ~ 50-100 base pairs in length. The V4 subregion, as an example, shows PCR amplification using primers that target the hypervariable region of interest (pink circles in full length 16*S* rRNA gene and pink arrows representing primer-binding sites) and the sequencing primer site (blue bottom arrow). Metagenomic sequencing using 454-pyrosequencing technologies utilise multiple hypervariable regions of the 16*S* rRNA gene to generate greater read lengths and sequence coverage in microbial identification (Petrosino et al., 2009).

The V1-V9 regions themselves have been shown to demonstrate different efficacies in terms of the species or genus phylotypes resolved within the microbial community analysed and their respective environments sampled. A study by Chakravorty *et al*., has shown that the V2 and V3 regions were the most effective at independent genus identification (Chakravorty et al., 2007); in particular is the application of the V3 hypervariable region in microbial community profiling PCR-DGGE mediated studies in which Yu, *et al*, (2004) showed that amplification of the V3 region within *rrs* genes generated better DGGE profiles than the other hypervariable regions amplified and analysed (Yu and Morrison, 2004).

In summary, culture-independent techniques have been an indispensable aid in describing the many microbial consortia in the biosphere of planet Earth and in us, human beings. In the following section we will demonstrate that we are collectively as a species intimately involved and indeed in contact with a characteristic microbiota incorporating bacteria, viruses, archaea, protozoans, and fungi (Foxman et al., 2008).

1.5 The bacterial ribosome and 16*S* rRNA

The constant manufacturing processes involved in protein biosynthesis occur on the ribosome and these play a key role in a bacterium's ability to proliferate in the environment, providing implications for bacterium-host interactions and crucially pathogenicity (Moss et al., 2007). This massive macro-molecular protein, 21 nm in size is comprised of both the 30*S* and 50*S* subunits which associate together via self-assembly forming the intact 70*S* bacterial ribosome (Ramakrishnan, 2002). Several publications of the three-dimensional structure of the ribosome have revealed the 30*S* and 50*S* subunits in great detail (Ban et al., 2000, Cate et al., 1999, Schluenzen et al., 2000, Schuwirth et al., 2005, Wimberly et al., 2000). Both subunits contain rRNA; the smaller 30*S* subunit comprising of 16*S* rRNA (1542 nt) and 21 ribosomal proteins (termed r-proteins), the larger 50*S* subunit is composed of two rRNAs, 23*S* (2904 nt) and 5*S* (120 nt) rRNA and 33 proteins (Noller and Nomura, 1996).

Within the *rrnB* operon (Fig. 1.4) downstream from both promoters P1 and P2, the 16*S* rRNA is encoded by the *rrs* genes; these express the formation of the small subunit (SSU) 16*S* rRNA ubiquitous in all bacterial species (one copy of this gene is always present in the bacterial genome — although copy number can vary between bacterial species; e.g., model organisms such as *Bacillus subtilis*, *Clostridium perfringens*, *Escherichia coli*, and

Lactococcus lactis have been previously shown to possess 10 (both in *B. subtilis* and *C. perfringens*) (Garnier et al., 1991, LaFuci et al., 1986), 7 (Kiss et al., 1977), and 6 (Beresford and Condon, 1991, Tulloch et al., 1991) *rrn* operons respectively) (Kaczanowska and Rydén-Aulin, 2007). Assembly of the prokaryotic ribosome is extremely complex and an in depth discussion of its assembly and multiple functions is beyond the scope of this thesis.

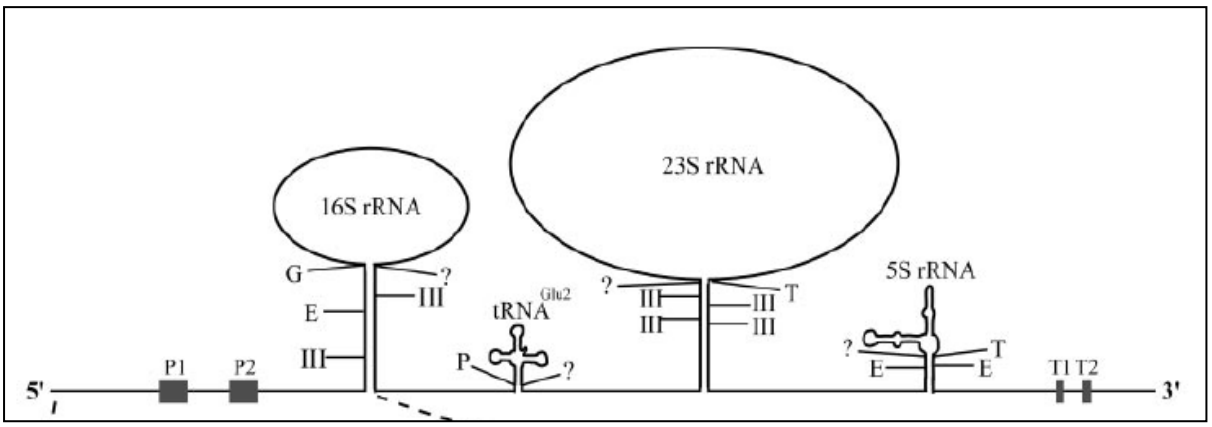


Fig. 1.4: The *rrnB* operon showing nucleolytic processing of the *rrnB* primary transcript. Downstream promoters P1 and P2 in the *rrnB* operon encode the formation of both 16*S* and 23*S* rRNA species and their associated ribosomal proteins (r-proteins) (not shown). Both the 5′ and 3′ sites are indicated in addition to the promoter and termination regions (P1, P2, T1, and T2 respectively). Also indicated are the processing sites of RNase III (III), RNase G (G), RNase E (E), RNase P (P), RNase T (T), and unknown RNases (?). These RNases process and chemically modify the pre-mature rRNA species in the positions shown above generating mature 16*S* and 23*S* rRNA species (Kaczanowska and Rydén-Aulin, 2007).

1.6 The human microbiome

In humans, estimates of the total amount of bacteria that colonise the gastrointestinal tract (GIT) outnumber our 'own' cells by up to 10-fold (Poxton, 2010). Acquisition of many bacterial taxa present in the gut is due to the early colonisation of these bacteria when the neonate first passes through the birth canal during labour (Palmer et al., 2007). The gut is not the only microbial ecosystem located in the human body, the skin, urogenital tract, and indeed the upper respiratory tract, have significant and complex bacterial communities (Gonzalez et al., 2011, Turnbaugh et al., 2007). These ubiquitous micro-organisms are an essential part of human evolution. In particular, mutual bacterial colonisation of the human mucosal surface interface forms a co-evolutionary relationship which benefits both the numerous bacterial species which inhabit these regions and the host; e.g., the upper respiratory tract (URT) and GIT plays host to ~ 600 and 1,000 unique species of bacteria respectively; identified as either permanent residents, others as transiently colonising these mucosal sites (Aas et al., 2005, Manson et al., 2008).

The Human Microbiome Project (HMP) of the National Institutes of Health (NIH) was launched in 2007 in a venture to begin to understand and characterise the human-associated microbiota and microbiome factors; i.e., collectively, the microbes that inhabit us and the genes in all of these micro-organisms (Gonzalez et al., 2011). We are only beginning to appreciate the full impact of the human microbiota in health and disease and the genetic diversity that encompasses the human microbiome in chronic diseases such as obesity (Turnbaugh et al., 2009), Crohn's disease (Eckburg and Relman, 2007) and other metabolic disorders (Spencer et al., 2011). Indeed micro-organisms are absolutely essential for human health offering beneficial effects such as the maintenance of pH in the oral and vaginal cavities, stimulation of the immune system, both innate and cellular, the prevention of invasion by PPMs, digestion of ingested foods through phase III metabolism processes, and finally providing nutrients vital to our health (Foxman et al., 2008). Most research is presently centred around bacterial diversity, whether this is through NGS or molecular fingerprinting methodologies, of the human microbiota, and several studies on bacterial diversity have been published on the GIT (Eckburg et al., 2005, Qin et al., 2010, Zoetendal et al., 1998), skin (Fierer et al., 2008, Gao et al., 2007), female urogenital tract (Brown et al., 2007, Zhou et al., 2007) URT (Charlson et al., 2010), and lungs (Hilty et al., 2010) of individuals amongst others. However, the human microbiome is not only comprised of

bacteria, other studies looking at both fungal and viral metagenomics (termed mycobiome(s) and virome(s) respectively) in human hosts have also recently been published looking at both diseased and non-diseased cohorts (Ghannoum et al., 2010, Reyes et al., 2010, Willner et al., 2009).

1.7 Microbial ecology

How does one make sense or comprehend this mass of genomic information contained within the human microbiome? In the GIT alone, it has been hypothesised that the entire gene complement of the intestinal microbiota was ~ 150 times larger than our own, many of these genes being uniquely encoded within this microbial assemblage (Qin et al., 2010). One way to tackle the increasing amounts of data and begin to model the numerous interactions within this abundance of microbes is to use microbial ecology hypotheses, i.e., looking at the microbial communities that make up the human microbiome from an ecological perspective.

Using a microbial ecological framework provides microbiologists and the biomedical community powerful schemes for hypothesis testing of the HMP datum (Dethlefsen et al., 2007, Robinson et al., 2010). Microbial community ecology arose from macro-ecology, i.e., the ecological frameworks used to study both the plant and animal kingdoms defined as "multi-species assemblages, in which organisms live together in an environment and interact with each other" (Knoopka, 2009). Using these frameworks enables microbial ecologists to better elucidate the way in which microbial communities that are part of the human microbiome contiguously interact within environmental niches inside the human body over space and time (Gonzalez et al., 2011). In addition, the investigation of the micro-environment within and its concurrent effects on the distribution and abundance of microbial species will provide a better understanding of the communities that inhabit us (Gonzalez et al., 2011). Adapting these theories and applying them to the microbial world has taken time, but nevertheless two studies in 2006 combined the metagenomic strategies with microbial ecology modelling for the datum generated; the first investigated the metagenomics of the microbial community in water from an iron mine in Minnesota (Edwards et al., 2006). Subsequently, a second study elaborated the metagenomics of rare members of several microbial communities using massively parallel pyrotag sequencing (Sogin et al., 2006). With this data, ecological measurements of richness, diversity, and similarity were all used for data analysis.

In essence, microbial diversity within sample types analysed, can be performed using three measures: alpha, beta, and gamma diversity indices defined by Whittaker in which (i), alpha diversity quantifies the richness of the species (number of taxa) in a niche; (ii), beta diversity compares the diversity between environments thus describing how disparate communities are structured in different niches; and (iii), gamma diversity in which both the alpha and beta diversity of communities from different landscapes and geographical regions are measured (Whittaker, 1972, Whittaker, 1969). In the microbial world, the definition of the term species is pragmatic (Amman et al., 1995). Conceptually speaking the term species encompasses those individuals as a group interbreeding within a species but isolated from other groups (i.e., other species) by genetic recombination barriers (Mayr, 1957). However, bacteria and some eukaryotes are asexual, contradicting this assumption, thus not forming ecologically distinct species defined genetically (Amman et al., 1995). Nevertheless, bacterial species are still defined by the use of operational taxonomic units (OTUs), i.e., the binning of coding nucleotide sequences from 16*S* rRNA genes to form species-level OTUs at a phylogenetic distance of 0.03, equivalent to 97 % nucleotide similarity against reference species sequence databases (Robinson et al., 2010). In addition to species diversity, other factors implicated in microbial ecology are biological divers of community structure, spatial patterning and temporal dynamics. All of these factors are hypothesised to affect the human microbiota.

1.8 Microbial biofilms

It has been recognised for some time now that microbes very rarely exist in isolation in natural, industrial, and medical habitats; microbes, and in particular bacteria, tend to form aggregated complex three-dimensional structures (Stickler, 1999). These aggregated bacterial cells are embedded within a hydrated matrix of extracellular polymeric substances (EPS) termed biofilms (Costerton et al., 1999, Donlan and Costerton, 2002). A polymicrobial biofilm therefore can be further defined as an eclectic assortment of fungi, bacteria, and viruses, attached to biotic and abiotic sites within the human host (Peters et al., 2012). Studies conducted on polymicrobial biofilm communities within the oral cavity enabled an initial understanding of the properties of biofilms.

Using bacteria as an example, the formation of biofilms is a result of an ancient adaptation (Hall-Stoodley et al., 2004) that prokaryotes utilise as a mode of growth in hostile environments, enabling the colonisation of new niches via dispersal mechanisms (Hall-

Stoodley and Stoodley, 2005, Mai-Prochnow et al., 2008, Purvedorj-Gage et al., 2005). Polymicrobial biofilm formation is through sequential attachment mechanisms termed co-aggregation, in which one species of microbe attaches itself to an appropriate substrate and then subsequently becomes a scaffold for other micro-organisms to facilitate adherence and biofilm formation (Rickard et al., 2003). Microbial biofilms are important both in healthy and diseased individuals due to several characteristics such as (i), phenotypic heterogeneity (ii), cell-cell communication via quorum sensing (QS), (iii), recalcitrance against antimicrobial compounds and (iv), resistance to both host phagocytic defences and antibodies (Hall-Stoodley and Stoodley, 2009). All of these properties enable biofilm infections to persist in the host despite administration of antimicrobial therapies and sustained host innate and cellular immune responses. Microbial biofilms are also beneficial to the host; specifically speaking, the abundance of conserved bacterial genera in the oral cavity has been previously shown using implanted sterile enamel chips into the mouths of human volunteers demonstrating the early colonization (~ 4 h) of *Streptococcus* spp. in addition to other genera such as *Actinomyces*, *Gemella*, *Granulicatella*, *Neisseria*, *Prevotella*, *Rothia*, and *Veillonella* (Diaz et al., 2006). Changes in this conservation of specific bacterial genera, whether over a rapid or slower time, has been hypothesised to promote disease-associated phenotypes within the human host; one example of this is the administration of broad-spectrum antibiotics which can severely affect the global bacterial populations within the community in the gut, i.e., reducing the protective properties of the polymicrobial commensals, thus allowing the proliferation of more invasive and resistant bacterial species in the site affected (Dethlefsen et al., 2008).

1.9 Polymicrobial infections

Just as polymicrobial biofilms are comprised not only of bacteria, but also viruses and fungi, polymicrobial infections can be defined as acute and chronic diseases caused by various combinations of viruses, bacteria, fungi, and also parasites (either protozoan or metazoan in origin) (Brogden et al., 2005). Further to this definition, polymicrobial infections are hypothesised to play a significant role within the human host and can manifest themselves via three distinct processes, although these processes could be all in unison (Brogden et al., 2005):

1. The generation of an environmental niche inside the human host due to the presence of one microbial species facilitates the colonisation of this niche by other PPMs.
2. When one species of microbe is present, this predisposes the host to subsequent colonisation by other micro-organisms.
3. Two or more non-pathogenic microbes are present and cause disease through synergistic effects in the host.

Some of the above concepts have been touched on recently, especially in relation to the theoretical concepts presented in microbial ecology, a key point here is that in polymicrobial infections, microbe-microbe interactions such as microbial interference can actually suppress the proliferation of other micro-organisms in the environmental niche generated, this interference can occur between PPMs or between probiotic microbes and pathogens (Bogaert et al., 2004). Synergistically, an environmental niche is generated by one microbe, as in periodontal disease, and this leads to subsequent colonisation by other PPMs (Palmer et al., 2001). Bacterial-viral synergism also plays a strong role in polymicrobial infections, for example, the isolation of human metapneumovirus (HPMV) in a range of debilitating respiratory tract conditions in conjunction with other viruses such as severe acute respiratory syndrome, with coronavirus, and bronchiolitis with respiratory syncytial virus (RSV) (Chan et al., 2003, Greensill et al., 2003). Infection with the measles virus also greatly pre-disposes the host to secondary infections with bacterial agents (Slifka et al., 2003); these secondary bacterial and viral infections can be attributed to the primary viral infection exhibiting and inducing an immunosuppressive effect within the host thus leading to, but not always, severe detrimental results. Additionally, infection with respiratory tract viruses can greatly pre-

dispose the host to colonisation or subsequent infection with a secondary micro-organism via destruction of the epithelial lining of the respiratory tract thus increasing bacterial adhesion and up-regulation of bacterial cell-surface receptors with the immunosuppressive properties of the viral infection which can lead to bacterial super-infections (Peltola and McCullers, 2004).

What is the basis for polymicrobial interactions in the human host and their origin? In all humans there is a co-evolutionary relationship between the host and their microbiome, this co-evolution has developed over time for thousands of years and has consequently resulted in species-specific physical and chemical interactions between microbes; these microbe-microbe interactions are employed due to the large variety and concentration of micro-organisms present and relatively minute amount of physical space available within us (Peters et al., 2012). Microbe-microbe interactions can be expressed variously within the host such as immunomodulation, augmented virulence phenotypes *in trans*, colonisation enhancement factors, cell-cell communication via QS, and contact-dependent attachment (Peleg et al., 2010).

Indeed, polymicrobial infections within human hosts are being increasingly recognised both in nosocomial and community settings as these several conditions imply: (i), human deficiency virus-acquired immunodeficiency syndrome (HIV-AIDS) patients in Africa are co-infected with malarial protozoan parasites and/or with *Mycobacterium tuberculosis* in addition to other micro-organisms (Lawn, 2004), (ii), bacterial vaginosis infection in women involves the increase in the mixed bacterial populations of *Gardnerella vaginalis*, Gram-positive anaerobic cocci and Gram-negative anaerobic rods such as *Peptostreptococcus* spp. and *Prevotella* spp. respectively, in addition to *Mycoplasma hominis* and *Ureaplasma urealyticum* as the *Lactobacillus* sub-populations rapidly decline (Hill, 1993, Persson et al., 2009, Pybus and Onderdonk, 1999), (iii), dental caries in the oral cavity is an extremely common condition affecting most of the adult population and younger children in which the hard enamel, dentin, and cementum surface surrounding the soft dental pulp is broken down (Holst, 2005). Breakdown of the hard dental surface is first by the creation of a polymicrobial biofilm — i.e., dental plaque — and secondly, the fermentation of carbohydrate sugars such as sucrose and fructose into lactic acid by several species of bacteria such as *Actinomyces viscosus*, *Lactobacillus acidophilus*, *Nocardia* spp., *Streptococcus mutans*, and the yeast

Candida albicans (Kinke et al., 2009, Minah and Loesche, 1977), (iv), chronic lower respiratory tract (LRT) infections in cystic fibrosis (CF) and chronic obstructive pulmonary disease (COPD) individuals are now thought to be polymicrobial in nature due to extensive studies, especially in CF where culture-independent techniques were employed (Erb-Downward et al., 2011, Harris et al., 2007, Hilty et al., 2010, Rogers et al., 2005a, Rogers et al., 2004, Rogers et al., 2003, Sethi et al., 2009, Sethi and Murphy, 2008, Sibley et al., 2006, Veeramachaneni and Sethi, 2006).

1.10 The respiratory tract

The respiratory system in humans is categorised into two separate components, the upper and lower respiratory tract, both operate in unison for the effective and efficient distribution of air, from the external environment into the internal environment of the lungs. The URT serves to warm and moisten the air when inspiration occurs protecting the LRT from PPMs and other foreign particles such as allergens, but also enabling for effective gaseous exchange. The LRT is responsible for the gaseous exchange of diatomic oxygen (O_2) in the alveoli to the peripheral blood capillaries that line the connecting tissues. Here O_2 diffuses across the membrane and enters erythrocytes, initially binding to haemoglobin in the process, that are circulating in the blood capillaries before releasing O_2 to the tissues and cells around and within the body where required. In humans, the process of respiratory function is an essential physiological process; it is the gaseous exchange of diatomic oxygen and carbon dioxide between blood and the external atmosphere (Bourke, 2002, Marieb and Hoehn, 2007).

The lung is the major interface between the internal and external environment of the human host, inspired air is abundant with opportunistic and pathogenic bacteria, viral and fungal micro-organisms (Bourke, 2002). Despite this constant exposure to potentially harmful particles, the health of an individual is maintained by sophisticated and complex host defence mechanisms (Stockley, 1998, Wilson et al., 1996). Defences such as the mucocilliary escalator which inhibits adherence and facilitates particle removal are dependent on bronchial epithelium integrity (Stockley, 1998). In addition, are antimicrobial agents such as lysozyme and lactoferrin that inhibit bacterial replication, secretory immunoglobulin A (IgA), and scavenging macrophages (Stockley, 1998).

1.10.1 Chronic respiratory tract diseases

Chronic respiratory tract diseases are of major global importance; they are increasing in incidence worldwide and are now classified by the World Health Organisation (WHO) as non-communicable diseases (NCDs) in addition to cardiovascular disease, cancer, and diabetes, collectively they account for the highest rates of mortality on a global scale than all other causes combined (Reardon, 2011). Chronic respiratory diseases encompass conditions such as CF, COPD, non-cystic fibrosis bronchiectasis, and asthma amongst others. Asthma and COPD present problems worldwide, both in terms of morbidity and mortality, but also in economic costs on healthcare resources. Asthma is a chronic inflammatory airway disease in which individuals suffer from exacerbations which result in chest tightness, wheezing, cough and dyspnoea, all symptoms associated with airway obstruction (Jarjour and Kelly, 2002). In the Western world, asthma is the most common chronic respiratory disease affecting 10 % of adults and 30 % of children, in the U.S. alone, 1.75 million asthma-related emergency department visits and 456 000 asthma-related hospitalisations were recorded contributing towards one-third of the total $14.7 billion annual U.S. asthma-related health care expenditure (Akinbami et al., 2011, American Lung Association). Chronic obstructive pulmonary disease is primarily induced by cigarette smoking and is characterised by irreversible airflow limitation and chronic inflammation in the LRT (Pauwels et al., 2001). COPD is an increasingly leading cause of morbidity and mortality globally, but woefully under-recognised (Pauwels and Rabe, 2004); from 1990 to 2020, the 30 year projection of COPD ranking will be a rise from sixth to third most common cause of mortality and fifth in the cause of disability worldwide (Lopez and Murray, 1998). Economically, in the U.K., £500 million is spent annually through direct healthcare costs relating to COPD in addition to 24 million working days also wasted (British Lung Foundation, 2006).

Cystic fibrosis is the most common recessive autosomal genetic disorder affecting individuals of Caucasian descent, ~ 1:2,500 children are afflicted with the condition from birth and ~ 70,000 adults and children are thought to be affected with CF worldwide (Cystic Fibrosis Foundation, World Health Organization, 2002) Genetically, CF is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene located on the long arm of chromosome 7, encoding for a membrane-bound cyclic-adenosine monophosphate (cAMP)-regulated chloride channel (Kerem et al., 1989, Riordan et al., 1989, Rommens et al., 1989). Mutations of the *CFTR* gene vary tremendously, and > 1,500

mutations and sequence variants have been identified presently and deposited into the Cystic Fibrosis Genetic Analysis Consortium. Although many of these mutations are rare, only four occur at > 1 % frequency, in particular, the most frequent mutation F508del exists in the region of DNA within the *CFTR* gene that encodes for one of two nucleotide-binding domains (NBDs), i.e., NBD1 (Döring and Ratjen, 2005). The mutation in NBD1 results in abnormal transport of chloride and sodium ions affecting water transport across mucus-rich epithelial tissues inducing systemic pathophysiological effects in CF individuals where the *CFTR* gene is expressed in various organs such as the respiratory, gastrointestinal, and reproductive tracts, the pancreas, and liver (Tizzano and Buchwald, 1995). Physiological dysfunction in the respiratory tract is the leading cause of morbidity in CF individuals as ~ 90 % die of respiratory failure due to this *CFTR* defect consequently leading to several cascading factors; dehydration of the airway mucous, reduced mucocilliary clearance, mucous retention, and predisposition of the CF patient to recurrent chronic lung infections (Boucher, 2002, George et al., 2009).

One of the main defining clinical features in CF and COPD is bronchiectasis; an abnormal dilatation of the bronchi and bronchioles due to repeated cycles of respiratory tract infection and subsequent inflammation (Barker, 2002, O'Donnell, 2008). Bronchiectasis is a highly debilitating disease which can cause premature mortality and chronic morbidity in some instances due to severe pulmonary infections and lung function impairment (Alzeer et al., 2007, King et al., 2005). The paradigm relating to chronic infection in bronchiectasis is a vicious circle of continual transmural infection and inflammation followed by mediator release, the retention of inflammatory secretions and micro-organisms in the LRT cause further lung tissue damage thus accelerating pulmonary obstruction and recurrent chest infection (Cole, 1986). Patients with CF present with a very severe form of bronchiectasis, but increasingly, other individuals are now being diagnosed with an idiopathic variant of this disease, termed non-CF bronchiectasis (nCFBR). The term idiopathic arises from the initial diagnosis of nCFBR in many patients in which the aetiology of the disease is unknown. The increasing frequency in the diagnosis of nCFBR is due to the widespread use of high-resolution chest computed tomography (HRCT) scanning (Cohen and Sahn, 1999), although the prevalence of nCFBR globally is unknown (O'Donnell, 2008). However, a recent study conducted in the U.S. alone revealed that 110,000 individuals may be receiving treatment for

nCFBR with an annual cost to the U.S. healthcare system of $630 million respectively (Weycker et al., 2005).

Most, if not all, individuals who are afflicted with asthma, COPD, CF, and nCFBR suffer from punctuated episodes of exacerbations, i.e., a worsening of symptoms which induce airway obstruction in these patients clinically presented as dyspnoea, cough, chest pain, and wheeze. Exacerbations can be triggered by several factors, including a secondary infection of both the URT and LRT of several aetiologies, but they also vary in their severity and frequency in the chronic respiratory disease briefly described above. In summary, chronic respiratory tract diseases play a significant role in the morbidity, mortality, and economies not only in the Western world, but increasingly in the developing world.

Cough, breathlessness, wheeze, chest pain, and sputum production, all major symptoms of respiratory disease may be indicative of acute or chronic respiratory tract infection (Leach, 2007). As the respiratory tract is a continuous entity, via the nose to the alveoli, respiratory tract infections (RTI) are distinguished by diagnoses of the 'preferred focus' of the infectious agent, either in the URT or LRT, although there can be at times ambiguity concerning this issue; for instance, the parainfluenza virus can infect the nasopharynx, larynx, trachea, and occasionally the bronchi and bronchioles resulting in the common cold, laryngotracheitis (croup), bronchitis, bronchiolitis, or pneumonia respectively (Goering et al., 2008). The aetiology of respiratory tract infection may be bacterial, viral or fungal in origin. Some patients may already present with an underlying lung disease such as nCFBR, CF, and COPD, of which infection in the upper and lower respiratory tract is a secondary disease, but nevertheless a critical contributing factor to the morbidity and mortality of the individual in question. For the purposes of the investigative work contained herein, the author will focus primarily on lower respiratory tract infections in non-CF bronchiectasis and COPD.

1.10.2 Respiratory tract defence mechanisms

Approximately 14,000 L of air is processed on a daily basis by the human lungs, of which contact between the external and internal environments is in constant flux, the integrity of the thin alveolar membrane must be maintained at all times to ensure efficient O_2 and CO_2 gaseous exchange within the host; thus the recognition, and conduct, of particulate matter without resulting in excessive inflammation is critically important (Valdivia-Arenas et al., 2007). The fragile alveolar epithelial layer itself is protected by non-specific factors such as

the cough reflex mechanism, ciliary beat, and mucocilliary escalator, in addition to several complex host innate defences comprising of both pulmonary cellular and humoral elements. Pulmonary innate cellular components are mediated by the airway and alveolar epithelial cells, but also leukocytes, both resident and recruited, including natural killer cells (NKCs), dendritic cells (DCs), cytotoxic T cells, macrophages, and neutrophils; antimicrobial products secreted into the epithelial lining fluid such as collectins, defensins, lysozyme, lactoferrin, secretory IgA, and cathelicidins are all constituents of the pulmonary humoral innate system (Zass and Schwartz, 2005). All of the above factors mitigate a collective role in the innate immune system including initial defence mechanisms against micro-organisms and clearance, but also crucially, subsequent activation of the adaptive immune system (Medzhitov and Janeway, 1997).

Recognition of PPMs in the respiratory tract is achieved by cell-surface conserved signature pathogen-associated molecular patterns (PAMPs) which are then mediated in turn by pattern-recognition receptors (PRRs) lining the airway epithelium, present in secretory components of the innate immune system and the cell-surface of leukocytes. Patter-recognition receptors are able to facilitate recognition of micro-organisms because of several common characteristics in that PAMPs are highly conserved and therefore critical to micro-organism subsistence, PPRs themselves are expressed constitutively within the host thus enabling detection of pathogens irrespective of life-cycle stage (Akira et al., 2006). The goal in the recognition of PPMs via PAMP-PPR mediation in the respiratory tract is two-fold; (i), to allow effective and efficient recognition of said PPMs by macrophages consequently leading to phagocytosis and induction of inflammatory mediators (Bals and Hiemstra, 2004), and (ii), to allow for discrimination between non-pathogenic and pathogenic elements that may come into contact with the airway epithelium in which the high specificity of PAMP-PPR interactions confer for these actions (Zass and Schwartz, 2005).

Toll-like receptors (TLRs) are an evolutionary conserved group of type I integral membrane glycoproteins characterised by their extracellular leucine-rich-repeat (LRR) motifs and COOH-terminal cytoplasmic signalling domains homologous to the interleukin 1 (IL-1) receptor in which the primary function of TLR molecules is that of PRRs (Baus and Fenton, 2004, Bowie and O'Neil, 2000). Twelve mammalian TLRs to date have been identified in mammalian hosts, TLR-2 and TLR-4 are the most extensively studied of the TLR families,

but all recognise related PAMPs from viral, bacterial, and fungal origins via highly specific binding to microbial ligands mediated by the variable LRR domain and intracellular signalling carried out by the conserved COOH-terminal intracellular tail (Armstrong et al., 2004). For example, TLR-2 and TLR-4 have all been shown to primarily bind to lipoteichoic acid (LTA) and lipopolysaccharide (LPS), both constitutive components of bacterial Gram-positive and Gram-negative cell wall composition respectively (Akira et al., 2006, Bals and Hiemstra, 2004, Zass and Schwartz, 2005); in addition, TLR2 has also been shown to bind to mycobacterial lipoarabinomannan, suggesting a defensive role in *Mycobacterium tuberculosis* infection and other atypical mycobacterial agents (Drennan et al., 2004, Reiling et al., 2002, Weiland et al., 2004). Molecular display of TLRs is not only limited to the respiratory tract epithelial cells and leukocytes but also DCs, B cells, and T cell subtypes, including extracellular (e.g., TLR-2 and TRL-4 bind to bacterial cell wall components LTA and LPS as previously discussed) and intracellular expression (e.g., TLRs 3 and 9 utilise viral nucleic acid double-stranded RNA (dsRNA) and DNA as receptors respectively (Alexopoulou et al., 2001, Hochrein et al., 2004, Tabeta et al., 2004)) modes (Akira et al., 2006). Constant modulation of TLR expression is an essential governing factor of the innate immune response if the host is effectively able to counter-attack insults by PPMs and respond to environmental stresses such as allergens thus inducing an inflammatory cascade and the release of components that comprise the adaptive immune response such as cytokines and chemokines (Zass and Schwartz, 2005). Indeed, as has been previously elucidated, an inflammatory cascade in the LRT in response to tissue architecture insults can in fact be occasionally detrimental to the host as seen in asthma, COPD, nCFBR, and CF respiratory conditions in which the innate immune response plays a pivotal role in the governance of these disease phenotypes.

1.11 Aims and objectives of research undertaken

The primary goal of the investigative work undertaken was to characterise the microbial communities in two chronic obstructive respiratory disease patient cohorts whilst also inferring the optimum DNA polymerase enzyme to perform the culture-independent analysis using an environmental matrix (sandy soil). Determination of a suitable DNA polymerase will aid in the subsequent investigation and characterisation of the polymicrobial communities of the lung microbiome. Using PCR-DGGE technique analysis both in cross-sectional patient cohorts presenting with nCFBR and COPD we aim to examine the effects of bacterial and fungal communities in the LRT and their relation to intrinsic and extrinsic factors such as pulmonary function, patient gender, height, and antimicrobial therapy, etc.

Chapter Two: Materials & methods

2.1 DNA polymerase enzymes and microbial diversity

2.1.1 Sandy soil sampling strategy

Soil samples were taken from dunes located on Longsands Beach, Tynemouth, North East England (55^0 1' 8.66'' N, 1^0 25' 36.23'' W) in October 2008. The sand dunes themselves had typical plant cover for the area, consisting mainly of Marram grass (*Ammophila* spp.). Five plots (1.5 m x 0.5 m) were created 1 m (± 0.25 m) up the dune. Within each of the five plots sandy soil was taken from five randomly placed sub plots at a depth of 20 cm. The sandy soil was then combined to create one sample per plot before being stored at -80 °C.

2.1.2 DNA extraction from sandy soil

Microbial genomic deoxyribonucleic acid (gDNA) extraction from sandy soil was performed using a MO BIO PowerSoil™ DNA Isolation Kit. To the PowerBead Tubes provided in the kit, 0.25 g of soil was added before being gently vortexed. Sixty µL of Solution C1 was then pipetted and mixed in the PowerBead tubes by inversion following addition of the lysis buffer. PowerBead Tubes were then vortexed (Vortex-Genie2™) at maximum speed for 10 min; these were secured horizontally to the vortexer using the MO BIO Vortex Adaptor tube holder. Following this, the PowerBead tubes were then centrifuged at room temperature for 1 min at 10 000 × *g* in which the supernatant was then transferred to a clean sterile 2 mL microfuge tube. Two hundred and fifty µL of Solution C2 was then added to the supernatant. This supernatant was then vortexed for 5 s and incubated for 5 min at 4 °C before being centrifuged at 10 000 × *g* at room temperature for 1 min to form a cell pellet. Avoiding the cell pellet, 600 µL of supernatant was transferred to a clean, sterile 2 mL collection tube in which 200 µL of Solution C3 was added and then vortexed briefly. An incubation time at 4 °C for 5 min followed before the collection tubes were centrifuged at 10 000 × *g* at room temperature for 1 min.

Again, transfer of the supernatant (up to 750 µL in volume) to a clean 2 mL collection tube was performed before the addition of 1.2 mL Solution C4 to the transferred supernatant

(ensuring at the same time that the Solution C4 supernatant mix did not exceed the rim of the 2 mL collection tube). Approximately 650 µL was then loaded onto a Spin Filter and centrifuged at 10 000 × *g* for 1 min at room temperature after which any flow through liquid was discarded. This Spin Filter process was repeated twice more (i.e., a total of three loads was required to pass through the Spin Filter). Five hundred µL of Solution C5 (ethanol wash solution) was added before centrifugation at 10 000 × *g* for 1 min at room temperature in which the flow through liquid was discarded from the 2 mL collection tube. Centrifugation at 10 000 × *g* was carried out again for 1 min at room temperature for effective removal of any residual ethanol wash solution (these trace amounts can interfere with many downstream DNA applications). After removal of Solution C5, the Spin Filter was placed into a clean sterile 2 mL collection tube and 100 µL of Solution C6, an elution buffer (10 mM Tris), was added to the centre of the white filter membrane releasing the previously bound gDNA from the silica Spin Filter membrane. The final step was to centrifuge the 2 mL collection tube at 10 000 × *g* for 1 min at room temperature after which the released gDNA (50 µL in volume) was collected. The Spin Filter was discarded and the extracted gDNA was stored at -80 °C.

2.1.3 Bacterial community detection PCR

Bacterial community detection by polymerase chain reaction (PCR) in all five soil samples was performed by amplification of the V3 hypervariable region within the bacterial 16*S* rRNA gene using extracted gDNA as template. Primers V3F-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and V3R (5′- ATT ACC GCG GCT GCT GG-3′) were used producing amplicons of 16*S* rDNA regions of different bacterial species in the soil community as previously shown (Muyzer et al., 1993). Amplification of the 16*S* rDNA V3 regions corresponded to positions 341 to 534 in *Escherichia coli*. Forward primer V3F-GC has an additional 40-bp GC-rich nucleotide sequence (GC-clamp) attached to its 5′ end allowing for higher sensitivity in detection of bacterial genomic DNA sequence variation in denaturing gradient gel electrophoresis (DGGE) processing and greater PCR fragment stability (Sheffield et al., 1989). Addition of bovine serum albumin (BSA) was also introduced into the PCR as this has been previously shown to relieve inhibitory substances such as humic acids which are present in soil and other environmental samples (Kreader, 1996).

Positive and negative controls were set up in all PCR experiments. Positive control bacterial gDNA was extracted from a pure culture of *Haemophilus influenzae* (NCTC (National Collection of Typed Cultures) no. 11931 (generously donated by Prof. J.D. Perry, Freeman Hospital, Microbiology Department, Newcastle upon Tyne, U.K.)) for confirmation of V3 region amplicons. A negative control set up was for the determination of any environmental contaminants that may be present during PCR. Five different DNA polymerases (pols) were implemented for enzymatic amplification of the V3 hypervariable region: Platinum® *Pfx* (Invitrogen™), *TaKaRa Ex Taq*™ Hot Start (TAKARA BIO INC.), AmpliTaq® (Applied Biosystems™), GoTaq® Hot Start (Promega), and *Taq* (New England BioLabs®, Inc.) respectively. All DNA polymerases used generated technical replicates for each sandy soil sample, 3 × 50 µL vials of amplicons per pol, eventually yielding 15 batches of amplicons altogether for each sandy soil sample analysed by DGGE. Common to the entire repertoire of *Taq* DNA pols executed here was a touchdown protocol (commonly referred to as touchdown PCR), and a manual and automatic hot start (HS). Touchdown PCR reduces the formation of spurious by-products, created in the amplification process, by increasing the expected initial annealing temperature by 10 °C (Don et al., 1991); i.e., 65 °C as opposed to 55 °C in this instance and then reducing the annealing temperature by 0.5 °C per cycle in PCR. Automatic HS DNA pols such as *Ex Taq*™ HS employ an antibody bound enzyme in which the activation temperature (e.g., 97 °C) results in release of the previously bound pol inhibiting not only spurious PCR product formation before initiation, but also minimising non-specific annealing primers to non-target DNA regions. Manual HS protocols execute a pol not bound to an antibody, but instead involve manually pipetting the pol at a specific volume and temperature (i.e., 80 °C) into the incubated sample once initial DNA template denaturation has been completed (i.e., 97 °C for 5 min). Introduction of DNA pols as a manual HS into PCR mirrors the purposes of automatic HS DNA pols. In summary, all of the above enzymes required different PCR conditions for satisfactory amplification of the V3 rDNA PCR fragments. These conditions were programmed into a thermocycler (Eppendorf ***Mastercycler gradient***) according to the manufacturer's recommendations for each DNA pol used in PCR. These conditions are described below.

2.1.3.1 V3 rDNA amplification using AmpliTaq™ DNA polymerase

The PCR performed using AmpliTaq® (Applied Biosystems™) pol involved the use of a manual HS and a touchdown protocol on a ***Mastercycler gradient*** (Eppendorf). PCR reagents per reaction were added as follows: 0.5 µM primers (both forward (V3F-GC) and reverse (V3R)), 1× GeneAmp® Buffer (Applied Biosystems™), 0.3 mM each dNTP (New England BioLabs®, Inc.), 2 mM $MgCl_2$ (SIGMA® Life Science), 10 µg/µL BSA (Promega), 1.25 U AmpliTaq® and 2 µL of DNA template made up to 50 µL with sterile 18.2 Ω H_20 (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). Samples were incubated for 5 min at 97 °C executing an initial denaturation step of template DNA before holding at 80 °C indefinitely to allow a manual HS via introduction of AmpliTaq® pol into the sample; then 96 °C for 1 min, 65 °C for 1 min, with a reduction of 0.5 °C per cycle until touchdown at 55 °C. Primer extension lasted for 30 s at 72 °C and this was repeated for 20 cycles. An additional fifteen cycles was carried out at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final extension time of 72 °C for 30 min. This extended final DNA elongation step was implemented to reduce the formation of artifactual double bands which can be problematic in microbial community profiling techniques such as DGGE (Janse et al., 2004).

2.1.3.2 V3 rDNA amplification using *Ex Taq*™ Hot Start DNA polymerase

Unlike the above conditions, PCR fragments amplified using *Ex Taq*™ HS (TAKARA BIO INC.) pol utilised an automatic HS and a touchdown protocol on a ***Mastercycler gradient*** (Eppendorf). PCR reagents per reaction were added as follows: 0.5 µM primers (both forward (V3F-GC) and reverse (V3R)), 1× Ex Taq Buffer (TAKARA BIO INC.), 0.2 mM each dNTP (TAKARA BIO INC.), 1 mM $MgCl_2$, 10 µg/µL BSA (Promega), 1.25 U *Ex Taq*™ HS and 2 µL of DNA template made up to a total volume of 50 µL with sterile 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). Samples were incubated for 5 min at 97 °C releasing antibody bound polymerase *Ex Taq*™ HS, then at 96 °C for 1 min, 65 °C for 1 min, with a reduction of 0.5 °C per cycle until touchdown at 55 °C. Primer extension lasted for 30 s at 72 °C and this was repeated for 20 cycles. An additional fifteen cycles was carried out at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final extension time of 72 °C for 30 min.

2.1.3.3 V3 rDNA amplification using GoTaq® Hot Start DNA polymerase

Amplification of V3 rDNA using GoTaq® HS (Promega) pol was carried out with both an automatic HS and touchdown conditions on an Eppendorf ***Mastercycler gradient***. PCR reagents per reaction were added as follows: 0.5 µM primers (both forward (V3F-GC) and reverse (V3R)), 2× Colorless GoTaq® Buffer (Promega), 0.3 mM each dNTP (New England BioLabs®, Inc.), 1 mM $MgCl_2$ (Promega), 10 µg/µL BSA (Promega), 1.25 U GoTaq® HS and 2 µL of DNA template made up to 50 µL with sterile 18.2 Ω H_20 (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). Samples were incubated for 5 min at 97 °C, releasing antibody bound polymerase GoTaq® HS, then at 96 °C for 1 min, 65 °C for 1 min, with a reduction of 0.5 °C per cycle until touchdown at 55 °C. Primer extension lasted for 30 s at 72 °C and this was repeated for 20 cycles. An additional fifteen cycles was carried out at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final extension time of 72 °C for 30 min.

2.1.3.4 V3 rDNA amplification using Platinum® *Pfx* DNA polymerase

Platinum® *Pfx* (Invitrogen™) pol conditions differed from the *Taq* DNA pol enzymes employed here as no touchdown PCR was carried out. However, an automatic HS was still performed. PCR reagents were added as follows: 0.5 µM primers (both forward (V3F-GC) and reverse (V3R)), 2× *Pfx* Amplification Buffer (Invitrogen™), 0.5× *Pfx* Enhancer Solution (Invitrogen™), 0.3 mM each dNTP (New England BioLabs®, Inc.), 1 mM $MgSO_4$ (Invitrogen™), 10 µg/µL BSA (Promega), 1.25 U Platinum® *Pfx* and 2 µL of DNA template made up to 50 µL with sterile 18.2 Ω H_20 (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). Samples were incubated at 94 °C for 4 min initialising DNA template denaturation and release of bound Platinum® *Pfx* pol. Following this, 35 cycles at 97 °C for 30 s, 65 °C for 30 s, and 68 °C for 30 s were performed with a final elongation step at 68 °C for 30 min.

2.1.3.5 V3 rDNA amplification using *Taq* DNA polymerase

New England BioLabs® Inc., *Taq* pol amplification of V3 rDNA amplicons was executed on a ***Mastercycler gradient*** (Eppendorf) using a manual HS and touchdown PCR. PCR reagents per reaction were added as follows: 0.5 µM primers (both forward (V3F-GC) and reverse (V3R)), 1× Standard *Taq* Reaction Buffer (New England BioLabs® Inc.), 0.3 mM each dNTP (New England BioLabs®, Inc.), 2 mM $MgCl_2$ (SIGMA® Life Science), BSA

(Promega), 5 U *Taq* pol and 2 µL of DNA template made up to 50 µL with sterile 18.2 MΩ H_20 (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). Samples were incubated for 5 min at 97 °C executing an initial denaturation step of template DNA before holding at 80 °C indefinitely to allow a manual HS via introduction of *Taq* pol into the sample; then 96 °C for 1 min, 65.8 °C for 1 min, with a reduction of 0.5 °C per cycle until touchdown at 55.8 °C. Primer extension lasted for 30 s at 72 °C and this was repeated for 20 cycles. An additional fifteen cycles was carried out at 94 °C for 1 min, 55.8 °C for 1 min, 72 °C for 3 min and a final extension time of 72 °C for 30 min.

2.1.4 Agarose gel electrophoresis of V3 rDNA amplicons

PCR fragments amplified by all five different polymerases were run out and validated using agarose gel electrophoresis (1 % ($^{w}/_{v}$) (App. 1) stained with SYBR® Safe DNA gel stain (10 000× concentrate in dimethyl sulphoxide (DMSO)) (Invitrogen™). Five µL of PCR product, 2 µL 6× bromophenol blue (BPB), and 5 µL 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter) were mixed by trituration before a final volume of 10 µL was loaded into the appropriate gel well, in addition to 5 µL of Hyperladder 1 (200-10 000 molecular bp size markers (BIOLINE)) (App. 2). PCR products were electrophoresed at 120 mA constant (BIO-RAD, Basic PowerPac™) for 20 min in 1× TAE running buffer (tris-acetate-EDTA (ethylenediamine tetraacetic acid)). Visualisation of PCR products was done under ultra-violet (UV) light using a BIO-RAD Gel Doc 2000 system and Quantity One software (v. 4.6.6). Comparison of the molecular bp markers in Hyperladder I (BIOLINE) and the positive control confirmed the correct size of the amplicons for the V3 rDNA fragments produced by each DNA pol utilised, i.e., 233 bp. Hard copies of the electropherograms were generated using a Mitsubishi Video copy processor (model P91 attached to the Gel Doc 2000 system).

2.1.5 Denaturing gradient gel electrophoresis of the bacterial community

2.1.5.1 Assembly of parallel gradient gel sandwich

Before DGGE analysis of PCR fragments, appropriate gel sandwich and casting was required (described below) for accurate determination of electrophoretic data. To ensure proper alignment of parallel gradient gel sandwich, all plates (16 × 16 cm) and spacers (1.0 mm in width) were clean and dry before assembly and constructed on a clean laboratory bench surface. The large rectangular glass plate (termed long glass plate) was laid down first

before placement of the spacers in which a thin layer of silicone grease (glisseal® HV) was applied to the straight edge of the spacers facing inwards towards the large glass plate. Application of a thin layer of silicone grease prevents leaking of denaturing substances such as urea and formamide into the buffer reducing the formation of "down smiling" across the gel gradient in all lanes used, in particular the outermost lanes (Brinkhoff and van Hannen, 2001). The smaller glass plate (termed short glass plate) was then placed on top of the spacers, but aligned so that it was flush with the bottom edge of the long glass plate. Each screw located at the top of the sandwich clamps was turned counter-clockwise (i.e., to loosen the screws) before placement at the appropriate side of the gel sandwich with the locating arrows facing up and towards the glass plates. Attachment of the sandwich clamps involved both the long and short plates fitting into the correct notches in the clamp (attached one at time) before tightening the screws (finger-tight) enough to hold both plates in position. Following this, a thin layer of silicone grease was applied to the entire bottom length of the assembled gel sandwich sealing the critical area (the attached spacers and glass plates) which prevents leakage of gel solution during the casting of gels (Brinkhoff and van Hannen, 2001).

The gel sandwich assembly was then placed into the alignment slot of the casting stand with the short glass facing towards the author. Sandwich clamps were loosened to allow the insertion of the alignment card to keep spacers parallel to the clamps. Alignment of plates and spacers simultaneously was achieved by pushing inward on both clamps at the locating arrows whist at the same time pushing down on the spacers ensuring that the spacers and glass plates are flush against the sides of the clamps. Both clamps were tightened just enough to hold the sandwich assembly in place before removal of the alignment card. The sandwich assembly was then removed entirely from the casting stand to ensure that both the plates and spacers were flush at the bottom. Clamps were then tightened until finger-tight.

2.1.5.2 Casting of parallel denaturing gradient gel

Using the front casting slot the gel sandwich assembly was placed on top of a grey sponge with the short plate facing towards the author. Upon correct positioning, the handles of the camshafts were turned down to lock the sandwich assembly in place. This is in preparation for gel casting. Pieces of Tygon tubing cut into two lengths of 15 cm and an additional shorter piece cut at 9 cm was used to conduct the gel solution from the Y-fitting into the gel sandwich assembly. One end of the shorter 9 cm piece of Tygon tubing was attached to the

Y-fitting, the other end a luer coupling with a 19 gauge needle (BD Microlance™ 3). This needle was then inserted in between the short and long glass plates of the sandwich assembly located in the centre before being taped (parcel tape providing the most stable attachment) to the forward facing side of the long glass plate Two other luer couplings were also connected to one end of both long Tygon 15 cm tubing pieces in addition to both 30 mL syringes (BD). Each syringe labelled either HI (for the high density solution) and LO (for the low density solution) had attached a plunger cap, positioned exactly onto each syringe plunger "head". Both syringes were correctly positioned into a syringe sleeve (with volume gradations visible); lever attachment screw was in the same plane as the flat or back side of the sleeve. This alignment enabled effective and undisturbed delivery of the denaturing gradient gel solution from the Gradient Delivery System. The cam wheel on the Gradient Delivery System was rotated back to its starting position (i.e., clockwise) with the delivery volume set to 16 mL.

Two 50 mL centrifuge tubes (SARSTEDT) labelled either HI (for high density solutions) or LO (for low density solutions) were used for preparation of denaturing gel solutions in a fume hood (Morgan and Grundy (Unitform) Ltd.). One hundred µL of DCode™ dye (App. 1) was pipetted into the HI 50 mL centrifuge tube before the addition of (i), 20 mL of 55 % denaturing gradient solution (12 % acrylamide/bis-acrylamide of gel percentage) (App. 1), 216 µL of 10 % ($^{w}/_{v}$) ammonium persulphate (APS (SIGMA-ALDRICH®)) (App. 1), and then 21.6 µL of *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED (SIGMA-ALDRICH®)) before the 50 mL centrifuge tube was inverted several times to mix contents; and (ii), into the LO 50 mL centrifuge tube, 20 mL of 35 % denaturing gradient solution (12 % acrylamide/Bis-acrylamide of gel percentage) (App. 1) 216 µL of 10 % ($^{w}/_{v}$) ammonium persulphate (APS), and then 21.6 µL of TEMED before the centrifuge tube was inverted several times to mix contents. Using the 30 mL syringes, with 15 cm Tygon tubing attached, ~ 18 mL of each denaturing gradient solution was withdrawn from both the HI and LO centrifuge tubes and into the appropriately labelled syringe vials. Any air bubbles present (in particular in the LO syringe) were removed at this time by turning the syringe upside down and gently tapping the syringe against a hard surface in addition to pushing these solutions to the end of the tubing to remove any further air bubbles.

The two syringes were then placed into the Gradient Delivery System syringe holder on each side, both designated LO and HI density side, by insertion of the lever attachment screws into the lever groove. Following this, the 'free' ends of the 15 cm Tygon tubing from the 30 mL syringes were then slid over and attached to both 'free' ends of the Y-fitting ready for denaturing gradient solution distribution. The cam wheel of the Gradient Delivery System was then rotated in a counter clockwise direction at a steady flowing pace with two hands in a constant motion for delivery of the denaturing gel solution from the attached syringes into the gel sandwich assembly. Once dispensed to the top of the short plate, the 19 gauge needle with Tygon tubing still attached was removed and placed into a 500 mL beaker of hot water before careful insertion of a 16-tooth comb. The gel was then left to polymerise for 1 h before use. Both HI and LO syringes were removed from the Gradient Delivery System with Tygon tubing still attached and then using the syringes themselves were thoroughly rinsed with hot water from the 500 mL beaker to remove any excess denaturing gel solution. This was repeated several times and performed quickly after gel casting to avoid premature gel polymerisation taking place inside the syringes, Tygon tubing and Y-fitting. After gel polymerisation (1 h from the initial gel casting procedure), the 16-tooth comb was removed by pulling it straight up and slowly as not to disturb the cast wells within the gel. Distilled water (dH_2O) was finally dispensed across the top of the short plate to fill all of the wells ready for sample loading.

2.1.5.3 Assembly of the upper buffer chamber before electrophoresis of amplicons

After gel polymerisation, the gel sandwich was released from the casting stand by turning the camshafts 180° to the up position and pulling them both outward and placed to the side on the bench. Laying the inner core flat on a bench, the gel sandwich assembly was then placed into position so the locating pins on the core fitted into the grooves on the outside surface of the sandwich clamps at an approximate angle of 20 °. The gel sandwich was then locked into position against the inner core by pushing up against the locating pins with the end result being that the upper edge of the short inner glass plate was seated against the notches of the U-shaped gasket and the tabs of both sandwich clamps held securely against the latch assemblies on both sides of the inner core. These above steps were repeated once again in order to attach a second gel sandwich assembly on the reverse side of the inner core, except soil sample 5 replicates (SS5R), in which only one gel sandwich assembly was required for

DGGE analysis. In this instance a 'balance' DGGE plate was constructed using the same method as the gel sandwich assembly above, but without the addition of the 1 mm spacers.

2.1.5.4 V3 rDNA amplicon preparation and loading on DGGE apparatus

The electrophoresis reservoir tank was filled with ~ 7 L of 1× TAE running buffer (App. 1) to the "fill" line and pre-heated up to 65 °C (this was carried out whilst performing the gel sandwich assembly and gel casting steps as reaching the desired running temperature can take 1 to 1.5 h). A 5 °C increase than the actual DGGE running temperature allows for quicker DGGE experimental and sample loading running time. Following this, the DCode™ temperature control module was switched off, removed, and placed on the DCode™ lid stand. Five hundred mL of 1× TAE running buffer was then removed from the reservoir tank to allow filling of the upper buffer chamber of the inner core assembly using a plastic beaker. The inner core with attached gel assemblies was placed into the buffer chamber with the red button towards the right hand side and the black button along the left hand side of the system. Approximately 350 mL of 1× TAE running buffer from the plastic beaker was decanted into the upper buffer chamber of the inner core assembly whilst in the reservoir tank; any excess was then used to fill the reservoir tank running buffer to the "max" line indicated.

All amplicons synthesised by AmpliTaq™, *Ex Taq*™ HS, Go Taq® HS, Platinum® *Pfx*, and *Taq* DNA polymerases respectively were prepared for loading onto the DCode™ system as follows; PCR products were thoroughly thawed, pulsed (MSE MicroCentaur (SANYO)) and 15 µL of amplicons was mixed with 15 µL of 2× gel loading dye by trituration before being pulsed again. Thirty µL of amplicons and 2× gel loading dye mixture was then loaded into the appropriate wells present in both gel sandwich assemblies attached to the inner core (except SS5R in which a balance plate was attached to the inner core). In terms of loading strategy, all 3 technical replicate amplicons synthesised by each DNA pol enzyme in sandy soil samples 1-5 was loaded as follows using SS1R as an example: replicates A-C synthesised by *Taq*, replicates A-C synthesised by Go Taq® HS, replicates A-C synthesised by AmpliTaq™, replicates A-C synthesised by *Ex Taq*™ HS, and finally replicates A-C synthesised by Platinum® *Pfx* (Fig. 3.2A-E). This loading pattern was repeated for the remaining four sandy soil samples analysed by DGGE. The DCode™ temperature control module from the DCode™ lid stand was placed back on top of the electrophoresis tank, ensuring that the stirring mechanism fitted neatly into the correct socket, locking the core in

place, and the power, pump, and heater were all switched back on with a final adjustment of the initial running temperature; i.e., 60 °C as opposed to 65 °C.

2.1.5.5 DGGE of V3 rDNA amplicon technical replicates in sandy soil samples

When a running temperature of 60 °C was finally obtained, technical replicates of V3 rDNA GC-clamped PCR fragments, amplified by the five different DNA polymerase enzymes in each sandy soil sample, were analysed by DGGE using the DCode™ Universal Mutation Detection System (BIO-RAD) on a 35-55 % denaturing gradient for 4.5 h at a constant of 200 V (Basic PowerPac™, BIO-RAD). All gels were removed before staining with SYBR Green nucleic acid stain I (Invitrogen™) for 30 min on a shaking platform at 75 rpm (Edmund Bühler, KM-2). The staining solution was then decanted and any excess present on the gel(s) was rinsed off with distilled dH_20. Visualisation of denaturing gels was under UV light using a BIO-RAD Gel Doc 2000 system and Quantity One software (v. 4.6.6). Hard copies of DGGE bacterial community profiles from each sandy soil sample and pol type were generated using a Mitsubishi Video copy processor (model P91 attached to the Gel Doc 2000 system).

2.1.6 Statistical and ecological analyses of bacterial community profiling

2.1.6.1 DGGE profile capture and analysis

The BIO-RAD Gel Doc 2000 system was used to capture all of the DGGE profile images (i.e., a photograph taken where the gel is converted into a digital file (TDS 1-D scan file format) producing an electropherogram) produced from the assessment of the DNA pol enzymes and the analysis performed using the included Quantity One software (version 4.6.6). Initial DGGE profiles captured were then converted to an inverted electropherogram (i.e., black bands set against a white background producing a clearer image for band discrimination). From here, a rolling ball algorithm was selected manually to subtract any background interference so peak detection performed represented actual bands and not artifacts. Lanes within the DGGE profile were created manually and subsequently framed to address DGGE experimental nuances such as ‘smiling’ and ‘leaning’ by the creation of lane anchors that could be manipulated to centralize all of the bands produced in each corresponding lane. Band detection itself was carried out using Quantity One’s auto-detect software algorithm with lane width set to 4.100 mm preventing lane overlap. Subsequently, detected bands in the DGGE profile were assessed by eye and either removed or added using

the Create/Remove bands tool. Bands generated across all lanes were then matched using the Match command in Quantity One in which unique bands identified in all five gels were matched across different lanes. A distinct band that appeared in a lane or across several lanes is identified via matching as a unique band type generating a band number. This was required to ascertain the normalised relative band intensities in the gel. Following band matching within the gel image produced, both qualitative and quantitative data arising from the DGGE profile was exported via the All Lanes Report command. Exportation of this data contained the relative quantity, lane and band number variables respectively. These three variables were then manually inputted into *Microsoft*® Excel™ where a DGGE band matrix was subsequently created.

Using the DGGE band matrix, the total intensity of each lane was calculated by using the AutoSum formula in *Microsoft*® Excel™ in which the normalised band values per lane were derived by dividing the relative intensity of each band in a lane by the total intensity value yielding a value between 0-1. A value of 0 corresponded to no band being present, whereas a value of 1 indicated that particular band accounted for the total intensity derived in that respective lane (i.e., one band was present or observable from the initial gel analysis performed in Quantity One).

2.1.6.2 Shannon diversity index derivation

Detection of the bacterial community by the five DNA polymerase enzymes employed was also assessed by calculation of the Shannon diversity index (H') in each sample type. The Shannon diversity index is a biodiversity quantitative measure which derives both the species richness (number of bacterial taxa present) and evenness (J') (how equal the different taxa are numerically distributed) in a sample type. The H' was calculated by using the formula:

$$H' = -\sum (p_i \ln[p_i])$$

Where p_i is the normalised relative intensity of each band detected in the DGGE profile, calculated using *Microsoft*® Excel™ (see section 2.1.6.1), and ln the natural logarithmic number of the normalised relative intensity band value. The ln was firstly derived of all the normalised relative intensity band values in each lane before being multiplied by these same values. Secondly, the sum of all these values per lane was multiplied by -1 converting the negative numbers into a positive H′ score for each respective DNA pol technical replicate.

Species richness (*R*) (i.e., the number of bacterial taxa present) was derived from the number of bands produced per lane per DNA pol technical replicate.

2.1.6.3 Principal components analysis

Once all normalised values were deduced in all five DGGE profiles generated, they were then copied and pasted into the PAST (v. 1.85) software program (Hammer and Harper, 2001) and subjected to multivariate analysis using principal components analysis (PCA). All Eigenvalues (a measure of variation in each axis measured in PCA) were < 1 so the PCA scores generated were accepted. The PCA scores of the first two axes of variation, principal components 1 and 2 (PC1 is the *x*-axis whereas PC2 represents the *y*-axis) were then used to plot graphs for each sandy soil sample and DNA pol technical replicate represented. The PC1 and PC2 percentage of variation were then added together to deduce the cumulative percentage of variation explained in the outputs. The variance between the DNA pol assessed and their detection of the bacterial diversity in each sample type was deduced by inputting both PC1 and PC2 scores into Minitab® 15 (v. 15.1.30.0). Variances along the *x*-axis and *y*-axis corresponding to PC1 and PC2 were subjected to a one-way ANOVA (analysis of variance) (confidence level was set at 95 %) where a *P*-value was calculated indicating whether there was a significant difference or not between the five DNA pol employed in the diversity generated in all five sandy soil samples. Additionally, a Tukey's test was performed, a post ad hoc ANOVA to determine if amplicons generated by the DNA pol enzymes differed.

2.2 Bacterial and fungal standard organism ladders for semi-quantitative DGGE analyses in patient cohorts

2.2.1 Bacterial 16*S* standard ladder construction

Nine bacterial species were selected for 16*S* standard ladder (SL) construction as these were all associated with microbial infection in both cystic fibrosis (CF) and non-CF bronchiectasis (nCFBR) individuals. These bacterial organisms (table 2.1) were comprised of reference strains from the NCTC, Laboratory of Microbiology, University of Ghent (LMG) and fully identified wild type strains from the Freeman Hospital, Microbiology Department, Newcastle upon Tyne.

Table 2.1 Bacterial species utilised in 16*S* standard ladder construction.

Number	Species	Reference strain
1	*Haemophilus influenzae*	NCTC 11931
2	*Staphylococcus aureus*	NCTC 6571
3	*Ralstonia pickettii*	NCTC 11149
4	*Pseudomonas aeruginosa*	NCTC 10662
5	*Streptococcus oralis*	NCTC 11427
6	*Burkholderia cepacia* complex	LMG 17997
7	*Stenotrophomonas maltophilia*	NCTC 10257
8	*Pseudomonas fluorescens*	NCTC 10688
9	*Achromobacter xylosoxidans*	Wild strain FRH 604914

The method of 16*S* SL construction has been previously demonstrated in both cross-sectional and temporal patient cohorts in CF using PCR-DGGE technique (Nelson, 2011). Briefly, the above bacterial isolates, with the exception of *H. influenzae*, were all inoculated onto Luria-Bertani (LB) agar (App. 1) and incubated overnight at 37 °C under aerobic conditions excluding *S. oralis* (incubated anaerobically). *H. influenzae* was cultured on NADsens agar (App. 1) at 37 °C in a static incubator with 5 % CO_2 saturation overnight. Subsequently, colony morphologies were validated by eye to ensure pure growth before a single colony (excluding *H. influenzae*) was utilised to inoculate 10 mL of sterile LB broth (incubated overnight at 37 °C). Colonies of *H. influenzae* were inoculated into 10 mL of sterile NADsens broth and subsequently incubated overnight in a microaerophilic environment. Examination of broth cultures for pure growth by inoculating fresh agar plates with 10 µL of culture and incubation overnight (as illustrated above) was performed as a quality control procedure. Once pure growth was validated, broth cultures were then subsequently utilised for gDNA extraction (UltraClean® Microbial Isolation Kit) (MO BIO Laboratories, Inc.)).

Using the extracted gDNA as template from each pure bacterial isolate grown, the V3 region of the 16*S* rRNA gene was targeted as previously demonstrated (Muyzer et al., 1993, Nelson et al., 2010) with the identical conditions described in section 2.3.4 performed on a ***Mastercycler gradient*** (Eppendorf) thermocycler. PCR amplification of V3 rDNA amplicons

was verified by agarose gel electrophoresis (as previously described in section 2.1.4) before being subsequently run out for DGGE analysis (DCode™ Universal Mutation Detection System (BIO-RAD)) on a 32.5-60 % denaturing gradient (12 % acrylamide/bis-acrylamide of gel percentage) (App. 1). Two µL of pure V3 rDNA amplicons mixed with 2 µL 2× loading dye was required before 4 µL was loaded into each well. The V3 rDNA amplicons from each bacterial species were also pooled and run out for DGGE analysis (36 µL in total (18 µL PCR products of 9 different bacterial species and 18 µL 2× gel loading dye)). Electrophoresis of bacterial isolate V3 rDNA amplicons was performed at 200 V constant (Basic PowerPac™, BIO-RAD) for 4.5 h, stained with SYBR Green nucleic acid stain I (Invitrogen™), incubated for 30 min (75 rpm on a shaking platform (Edmund Bühler, KM-2)), de-stained briefly with dH_2O, before visualisation under UV light (BIO-RAD Gel Doc 2000 system and Quantity One software (v. 4.6.6)). Hard copies were generated as previously described. Pure V3 rDNA single bacterial strain PCR products amplified from the bacterial isolates listed above were to comprise the 16*S* SL. Execution of this 16*S* SL was essential for inter-comparison of the bacterial DGGE community profiles generated in all of the patient cohorts analysed enabling inter-gel alignment via these internal standards (Tourlomousis et al., 2010).

2.2.2 Fungal 28*S* standard ladder construction

2.2.2.1 Fungal isolates selection and collection

Seven fungal species were selected and utilised for the construction of the 28*S* SL for fungal community studies in all patient cohorts enlisted. Selection of the following was based on the implication of all species being opportunistic invasive respiratory pathogens in CF patients. Fungal species chosen were comprised of one spore-forming species, *Aspergillus fumigatus*, and five yeast-forming species: *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida parapsilosis*, *Exophiala dermatitidis*, and *Scedosporium apiospermum*. All fungal collections prior to gDNA extraction were generously donated by Mrs. Claire Rennison at the Freeman Hospital, Microbiology Department, Newcastle upon Tyne. The fungal isolates themselves were from clinical samples, identified in the Microbiology Department laboratory according to standard operating procedure (SOP) affiliated with the Freeman Hospital.

2.2.2.2 Extraction of fungal gDNA from isolates

Extraction of fungal gDNA was performed using an UltraClean® Microbial Isolation Kit (MO BIO Laboratories, Inc.) in a Class II safety airflow cabinet (envair Bio 2+) with amendments to the protocol. Fungal species were firstly inoculated in 100 mL of sterile Sabourad (OXOID) liquid broth (pH 7.0) (App. 1) before being incubated (SANYO) at 22 °C for 7 days. Following incubation, a tea spoonful of sterile glass beads (3 mm diameter) was dropped into each fungal culture species before being placed in an orbital shaker (New Brunswick Scientific Ltd.) for 10 min at 200 rpm, 37 °C. This step is critical as it aids in the rupture and softening of the previously hard mycelial wall (prominent in the spore-forming species *A. fumigatus*) seen after the first incubation step.

From the yeast cultures grown, 1.8 mL of microbial culture (broth liquid medium) was pipetted into a clean, sterile 2 mL microfuge tube and centrifuged at 10 000 × *g* for 1 min at room temperature before the supernatant was decanted. The tubes were then spun down again at 10 000 × *g* at room temperature generating a cell pellet. Any supernatant still present was removed by a pipette tip. For *A. fumigatus* fungal culture: using a clean pair of forceps (washed down with 70 % $^{v}/_{v}$ ethanol), a small piece of softened mycelia present at the top of the Sabourad broth was transferred to a clean sterile 2 mL collection tube, centrifuged at 10 000 × *g* for 1 min at room temperature before the supernatant was discarded. Again, this was spun down once more at 10 000 × *g* for 1 min at room temperature in which any supernatant still present was removed by pipette as in the yeast species above. Cell pellets were resuspended in 300 µL of MicroBead Solution then gently vortexed before transfer of resuspended cells to MicroBead Tubes. Fifty µL of MD1 Solution was then added to the Microbead Tubes.

Fungal cell preps (in MicroBead Tubes) were then heated at 70 °C for 10 min as an additional aid in fungal cell lysis. Following this incubation step, fungal cell preps were then vortexed at maximum speed for 10 min using the Mo Bio Vortex Adapter tube holder. After bead beating step, the 2 mL MicroBead Tubes were then centrifuged at 10 000 × *g* for 1 min at room temperature. The supernatant (~ 300-350 µL) was aliquoted into a clean sterile 2 mL collection tube in which 100 µL of Solution MD2 was then added. Microfuge tubes were gently vortexed for 5 s then incubated at 4 °C for 5 min. Following this incubation step, fungal cell preps were centrifuged at 10 000 × *g* for 1 min at room temperature. The entire

volume of supernatant (~ 450 µL) was then transferred to a clean sterile 2 mL collection tube, whilst avoiding the cell debris pellet. Solution MD3 was briefly shaken and 900 µL was aliquoted to the recently transferred supernatant and then vortexed for 5 s.

Seven hundred µL was then loaded into the Spin Filter and centrifuged at 10 000 × *g* for 1 min at room temperature twice. All flow through liquid following this centrifugation step was discarded. Three hundred µL of MD4 Solution was then added before centrifugation at room temperature for 1 min at 10 000 × *g*. Flow through liquid from Spin Filter was again discarded before repeating the above centrifugation step (10 000 × *g* at room temperature for 1 min). Spin Filter basket was then removed and placed into a new clean sterile 2 mL collection tube in which 50 µL of Solution MD5 was pipetted to the centre of the white filter membrane. Microfuge tubes containing Spin Filter baskets were then finally centrifuged at room temperature for 1 min, 10 000 × *g* to elute isolated fungal gDNA. The Spin Filter basket was discarded and fungal gDNA was stored at -80 °C.

2.2.2.3 PCR amplification of 28*S* rDNA segments

Amplification of all seven fungal isolates using extracted gDNA was used as template for PCR. Two conserved regions flanking a highly variable region within the 28*S* rRNA gene were selected as previously demonstrated for amplification of small segments of 28*S* rDNA (Sandhu et al., 1995). Universal fungal primers U1F (5′-GTG AAA TTG TTG AAA GGG AA-3′) and U2R-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG-3′) primer sets were executed, in addition to *Ex Taq*™ HS pol (TAKARA BIO INC.), to enzymatically amplify small segments of 28*S* rDNA (U1-U2 rDNA fragments). Both universal fungal primers both refer to co-ordinate numbers 403-422 (forward primer U1F) and 645-662 (reverse primer U2R) of the reference *Saccharomyces cerevisiae* 28*S* rRNA gene (Sandhu et al., 1995). As in the bacterial community detection PCR, addition of a GC-clamp was executed, however, this was attached to the reverse fungal primer (U2R-GC) to aid in genomic sequence variation detection in DGGE (Sheffield; 1989). The PCR of the fungal isolates was performed on a ***Mastercycler gradient*** (Eppendorf). PCR reagents for this reaction were added as follows: 0.5 µM primers (both forward (U1F) and reverse (U2R-GC)), 1× Ex Taq Buffer (TAKARA BIO INC.), 0.2 mM each dNTP (TAKARA BIO INC.), 1 mM $MgCl_2$, 10 µg/µL BSA (Promega), 1.25 U *Ex Taq*™ HS and 1 µL of gDNA template made up to a total volume of 50 µL with sterile 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra

Pure Water Systems, MILLIPAK® 0.22 µm filter). An automatic HS and touchdown PCR protocol was utilised in which the conditions were an initial denaturation step of 97 °C for 5 min, 94 °C for 1 min, 60 °C for 1 min with a reduction of 1.0 °C per cycle reaching touchdown at 50 °C and 72 °C for 1 min repeated for 10 cycles. An additional 25 cycles were performed at 94 °C for 3 min, 50 °C for 1 min, and 72 °C for 1 min. As previously introduced, a final extension time at 72 °C for 30 min was implemented in addition to the touchdown protocol (Don et al., 1991, Janse et al., 2004).

2.2.2.4 Agarose gel electrophoresis of 28*S* rDNA segments

The small pure 28*S* rDNA fragments from each fungal isolate were validated for amplification by running PCR products out on a 1 % ($^{w}/_{v}$) agarose gel stained with SYBR® Safe DNA gel stain (10 000× concentrate in (DMSO) (Invitrogen™). PCR fragments were then electrophoresed for 20 min at 120 mA constant (BIO-RAD, Basic PowerPac™) in 1× TAE running buffer with 5 µL of PCR product, 2 µL 6× BPB, and 5 µL 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter) mixed prior to 10 µL loaded into each well. Five µL of Hyperladder I (200-10 000 molecular bp-size markers (BIOLINE)) was also added, in which after electrophoresis amplicons migrated between the 200 and 400-bp marker sizes (amplicon size was 300 bp in length, including the GC-clamp attached to U2R-GC reverse primer) confirmed under UV light (Gel Doc 2000 system and Quantity One software (v. 4.6.6) (BIO-RAD). Hard copies of the electropherograms were generated using a Mitsubishi Video copy processor (model P91 attached to the Gel Doc 2000 system).

2.2.2.5 DGGE of U1-U2 rDNA PCR fragments

The amplification of 28*S* rDNA segments (termed U1-U2 rDNA) using PCR from the fungal isolates selected for 28*S* standard ladder construction were subsequently analysed by DGGE to validate individual fungal strain migration position and pooled amplicon migration positions; a necessity when the fungal communities were amplified in patient cohorts investigated in thesis. Fungal species standard ladder analysis was performed using the DCode™ Universal Mutation Detection System (BIO-RAD) where U1-U2 rDNA fragments from each fungal isolate were run out on a 40-60 % denaturing gradient (12 % acrylamide/bis-acylamide of gel percentage) (App. 1) in addition to a balance plate attached to the inner core. Four µL of each fungal strain and its amplicons were loaded into the

appropriate gel wells (2 µL pure fungal strain U1-U2 rDNA and 2 µL 2× loading dye). Additionally, all fungal species amplicons were pooled together (28 µL (14 µL PCR products of 7 different fungal species and 14 µL 2× gel loading dye)) and loaded onto the DGGE apparatus. All PCR fragments were then electrophoresed at 70 V constant (Basic PowerPac™, BIO-RAD) for 17 h, stained with SYBR Green nucleic acid stain I (Invitrogen™), incubated for 30 min at 75 rpm, (Edmund Bühler, KM-2) and de-stained briefly with dH_2O, before being finally visualised under UV light (BIO-RAD Gel Doc 2000 system and Quantity One software (v. 4.6.6)). Hard copies of the fungal 28*S* standard ladder were generated by using a Mitsubishi Video copy processor (model P91 attached to the Gel Doc 2000 system).

2.3 PCR-DGGE study of a cross-sectional cohort with non-CF bronchiectasis

2.3.1 Patient cohort selection and ethical considerations

Ethical approval was obtained from County Durham and Tees Valley research ethics committee. The inclusion criteria were adult out-patients attending a specialist bronchiectasis weekly clinic in North East England (Freeman Hospital, Newcastle upon Tyne) with a clinical diagnosis of non-cystic fibrosis bronchiectasis (nCFBR) diagnosed by high-resolution computed tomography (HRCT) scanning. Seventy patients (App. 3) were enrolled into the study in which all non-CF aetiologies were included with the related nCFBR condition. Information on bronchiectasis aetiology, patient sex, age, clinical status, forced expiratory volume in one second (FEV_1) and one second predicted (FEV_1% predicted) in addition to inhaled antibiotic therapy was collected. Patient cohort enrolled consisted both of clinically stable and exacerbated individuals. For clinical status an exacerbation was defined as per the British Thoracic Society 2010 Bronchiectasis guidelines in the presence of increased cough, malaise and increased sputum volume and purulence (Pasteur et al., 2010). Frequent exacerbators within cohort were defined as those patients who had greater than 3 episodes over this period (Pasteur et al., 2010). Aetiological designation was based upon the suggested protocols (Pasteur et al., 2001). Cystic fibrosis genotyping and/or sweat testing was undertaken as suggested by national guidelines.

2.3.2 Sputum sample collection and diagnostic microbiology

Spontaneously expectorated sputum samples were collected from an outpatient cohort of an adult nCFBR population. Samples were divided with one half being used for genomic DNA extraction (below) whilst the remainder underwent routine microbial culture using 10 µL aliquots of Sputasol (OXOID) treated and homogenised sputum according to the standardised Health Protection Agency methodology at the Department of Microbiology, Freeman Hospital, Newcastle-upon-Tyne (see App. 4 for standard operating procedure (SOP) regarding the selective media employed from this sample type). Information, from up to 10 years previously, on patient sex, age, clinical and microbiological status, FEV_1, and inhaled antibiotic therapy was collected. The definitions of Pasteur *et al*., (2001) were used to define persistent infection in study cohort.

2.3.3 DNA extraction from sputum clinical samples

All DNA extractions on sputum samples in patient cohort were performed using an UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) in a Microflow Peroxide Advanced Bio Safety Cabinet (Class I) at the Freeman Hospital, Microbiology Department. Before collection of extracted gDNA from sputum all samples were thawed thoroughly and then vortexed (Vortex-Genie2™) rigorously after addition of an approximate equal volume of sputolysin (if required as most sputum samples varied in their degree of purulence). The addition of sputolysin breaks down any mucus plugs that may be present in the sputum sample and aids in the creation of a more aqueous solution enabling the sample supernatant to pass through the 0.2 µm filter without obstruction.

Once mucus plugs in sample were dissolved, 1.8 mL from aqueous solution (i.e., the sputum sample) was aliquoted into a sterile 2 mL collection tube before being spun down at 10 000 × *g* (SIGMA® 12094 rotor) at room temperature for 1 min in a microcentrifuge (SIGMA®). The supernatant was discarded leaving behind another mucus plug. At this stage all sputum samples extracted for gDNA left behind varying amounts of mucus plugs. As a precautionary step, any mucus plugs which exceeded > 200 µL needed be to be aliquoted off to approximately < 200 µL to ensure unobstructed flow through 0.22 µm filter at a later stage. Following this the sample was spun down again at 10 000 × *g* to maximally concentrate and pellet microbial cells. Any supernatant present in sample was discarded. The cell pellet was re-suspended in 300 µL of MicroBead Solution containing salts and a buffer stabilising and

homogeneously dispersing the microbial cells present in the re-suspension solution prior to cellular lysis. After addition of MicroBead Solution re-suspended cells were gently vortexed to aid in microbial cell dispersion before being transferred to a MicroBead Tube. Fifty µL of MD1 solution (i.e., lysis buffer) was then added to re-suspended cells. The lysis buffer contains the anionic detergent sodium dodecyl sulphate (SDS) and other chaotropic salts (undisclosed in kit protocol, but these can be guanidine hydrochloric acid (HCl), guanidine thiocyanate, urea, and lithium perchlorate). Both chemical agents aid in cellular lysis, the former acting to breakdown fatty acids and cell membrane lipids via solubilisation; the latter, in addition to also breaking down the cellular membrane, plays an important role in the disruption of hydrogen bonds, van der Waals forces, and hydrophobic interactions.

All MicroBead Tubes were then secured horizontally using the MO BIO Vortex Adapter tube holder and subsequently vortexed at maximum speed for 10 min to release ssDNA from lysed cells. Recovery of gDNA from cell debris was achieved by centrifuging at room temperature the 2 mL MicroBead Tubes at 10 000 × *g* for 1 min in which the supernatant (now containing the gDNA) was transferred to a clean 2 mL microfuge tube. One hundred µL of MD2 Solution was added to the supernatant and then vortexed for 5 seconds before being incubated at 4 °C for 5 min. Solution MD2 precipitates both non-DNA and inorganic material present in proteins and cell debris. After incubation, the supernatant was then spun down at room temperature at 10 000 × *g* for 1 min.

Avoiding the pellet, as this contained non-DNA organic and inorganic materials (i.e., cell debris and proteins), the entire supernatant volume was transferred into an empty syringe vial (BD) with filter (PALL Gelman Laboratory, Acrodisc® Syringe Filter, 0.2 µm Supor® membrane, non-pyrogenic) attached before insertion of the syringe plunger. Once inserted, plunger was pushed down to filter sterilise the supernatant into an empty, clean 2 mL collection tube. Filter sterilisation of the supernatant ensured the removal of any tubercular agents that may be still present in the supernatant processed. Approximately 200-300 µL of supernatant volume was collected after this step.

Solution MD3 was shaken before use and then 900 µL of this was added to the filter sterilised supernatant and subsequently vortexed for 5 s. Solution MD3 creates a high salt concentration which is necessary for the binding of the gDNA to the Spin Filter in the following step. Seven hundred µL of supernatant was loaded into the Spin Filter and

centrifuged (room temperature) at 10 000 × *g* for 1 min before the flow through was discarded. The remaining supernatant was added to the Spin Filter and this process was repeated once more. Again, all flow through liquid was disposed of as the gDNA is selectively bound to the silica membrane in the Spin Filter device allowing impurities such as protein and polysaccharides to pass unobstructed in the flow through liquid. Three hundred µL of Solution MD4 (an ethanol based wash solution) was then added to further clean the gDNA bound to the silica membrane removing residues of salt and other impurities that may be still present in the previous step. All flow through liquid containing ethanol wash solution waste and contaminants was discarded as before. The Spin Filter was then spun down at 10 000 × *g* for 1 min at room temperature to remove any residual ethanol wash solution present (essential for a clean eluant). The Spin Filter basket was then placed in a new clean 2 mL collection tube before addition of 50 µL Solution MD5 (an elution buffer containing 10 mM Tris, pH 8) to the centre of the white membrane. The collection tube containing the Spin Filter was centrifuged at 10 000 × *g* at room temperature for 1 min eluting the gDNA bound to the filter membrane. The elution buffer solution MD5 allows this, as release of the bound DNA is permitted only in the presence of this elution buffer as it contains no salt (previously, DNA was bound to the silica Spin Filter membrane only in the presence of salt). Following elution of the gDNA (50 µL in volume) the Spin Filter was discarded and the gDNA was stored at -80 °C.

2.3.4 Bacterial community detection PCR

As previously described, the V3 hypervariable region of the 16*S* rRNA gene was targeted for enzymatic amplification using gDNA as template. Primer sets V3F-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and V3R (5′- ATT ACC GCG GCT GCT GG-3′) as previously shown (Muyzer et al., 1993, Nelson et al., 2010) were utilised to produce different V3 rDNA fragments amplified from multiple species of bacteria present in the clinical sputum samples. As before, a 40-bp GC-rich nucleotide sequence was attached to the 5′ end of the forward primer V3F-GC (Sheffield et al., 1989).

An automatic HS and touchdown PCR protocol (Don et al., 1991) was performed on a ***Mastercycler gradient*** (Eppendorf) with the PCR reagents per reaction added as follows: 0.5 µM primers (both forward (V3F-GC) and reverse (V3R)), 1× Ex Taq Buffer (TAKARA BIO

INC.), 0.2 mM each dNTP (TAKARA BIO INC.), 1 mM $MgCl_2$, 10 µg/µL BSA (Promega), 1.25 U *Ex Taq*™ HS pol and 1 µL of gDNA template made up to a total volume of 50 µL with sterile 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). Positive (extracted gDNA from a pure culture of *H. influenzae*, NCTC no. 11931) and negative controls were also set up to determine the correct size of the V3 rDNA fragments and to rule out any environmental DNA contamination. Samples were incubated for 5 min at 97 °C releasing antibody bound polymerase *Ex Taq*™ HS, then at 96 °C for 1 min, 65 °C for 1 min, with a reduction of 0.5 °C per cycle until touchdown at 55 °C. Primer extension lasted for 30 s at 72 °C and this was repeated for 20 cycles. An additional 15 cycles was carried out at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final extension time of 72 °C for 30 min as previously described (Janse et al., 2004).

2.3.5 Fungal community detection PCR

As previously described in the 28*S* standard ladder construction sections, in addition to the bacterial community, we also investigated the fungal community in the cross-sectional cohort. Using primer sets U1F (5′-GTG AAA TTG TTG AAA GGG AA-3′) and U2R-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG-3′) to target conserved regions containing hyper-variable regions within the 28*S* rRNA gene, small fragments of 28*S* rDNA were generated using gDNA as template (Sandhu et al., 1995, Nelson, 2011).

An automatic HS and touchdown PCR protocol was performed on an Eppendorf ***Mastercycler gradient*** apparatus. Reagents for this reaction were added as follows: 0.5 µM primers (both forward (U1F) and reverse (U2R-GC)), 1× Ex Taq Buffer (TAKARA BIO INC.), 0.2 mM each dNTP (TAKARA BIO INC.), 1 mM $MgCl_2$, 10 µg/µL BSA (Promega), 1.25 U *Ex Taq*™ HS (TAKARA BIO INC.) and 1 µL of gDNA template made up to a total volume of 50 µL with sterile 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). Conditions for the PCR were as follows: an initial denaturation step of 97 °C for 5 min, 94 °C for 1 min, 60 °C for 1 min with a reduction of 1.0 °C per cycle reaching touchdown at 50 °C and 72 °C for 1 min repeated for 10 cycles. An additional 25 cycles were performed at 94 °C for 3 min, 50 °C for 1 min, and 72 °C for 1 min, with a final extension time at 72 °C for 30 min implemented as previously described in addition to the touchdown protocol (Don et al., 1991, Janse et al., 2004). Positive and

negative controls were also conducted confirming the correct size of the fungal U1-U2 rDNA amplicons (using extracted gDNA from a pure culture of *A. fumigatus*) and validation of any environmental sources of contamination.

2.3.6 Agarose gel electrophoresis of both V3 and U1-U2 rDNA amplicons

Regions amplified from both the 16*S* and 28*S* rRNA genes in extracted gDNA, i.e., V3 and U1-U2 rDNA fragments, were confirmed by running PCR products on a 1 % ($^{w}/_{v}$) agarose gel stained with SYBR® Safe DNA gel stain (10 000× concentrate in (DMSO) (Invitrogen™). PCR fragments were electrophoresed for 20 min at 120 mA constant (BIO-RAD, Basic PowerPac™) in 1× TAE running buffer with 5 µL of PCR product, 2 µL 6× BPB, and 5 µL 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter) mixed prior to 10 µL loaded into each well. Five µL of Hyperladder I (200-10 000 molecular bp-size markers (BIOLINE)) was also added, in which after electrophoresis, positive confirmation of both the V3 and U1-U2 rDNA fragments was validated (233 and 300 bp in size respectively) under UV light (Gel Doc 2000 system and Quantity One software (v. 4.6.6) (BIO-RAD). Hard copies of the electropherograms were generated using a Mitsubishi Video copy processor (model P91 attached to the Gel Doc 2000 system).

2.3.7 Molecular fingerprinting of bacterial and fungal communities in patient cohort

2.3.7.1 DGGE of V3 rDNA amplicons

Molecular fingerprinting of V3 rDNA amplicons in cross-sectional patient cohort was carried out using a DCode™ Universal Mutation Detection System (BIO-RAD) run out on a 32.5-60 % denaturing gradient (12 % acrylamide/bis-acrylamide of gel percentage) (App. 1) with a balance plate attached to the inner core of the DCode™ system apparatus. PCR fragments generated from each clinical sample (30 µL (15 µL PCR product and 15 µL 2× gel loading dye) were loaded onto wells in the denaturing gel in addition to a 16*S* standard ladder (36 µL (18 µL PCR products of 9 different bacterial species and 18 µL 2× gel loading dye)). Electrophoresis of V3 rDNA amplicons was performed at 200 V constant (Basic PowerPac™, BIO-RAD) for 4.5 h, stained with SYBR Green nucleic acid stain I (Invitrogen™), incubated for 30 min (75 rpm on a shaking platform (Edmund Bühler, KM-2)), de-stained briefly with dH_2O, before visualisation under UV light (BIO-RAD Gel Doc

2000 system and Quantity One software (v. 4.6.6)). Hard copies of the bacterial community DGGE profiles in cross-sectional patient cohort were generated (Mitsubishi Video copy processor (model P91) attached to the Gel Doc 2000 system).

2.3.7.2 DGGE of U1-U2 rDNA amplicons

Fungal community analysis of nCFBR cohort was performed using the DCode™ Universal Mutation Detection System (BIO-RAD) on a 40-60 % denaturing gradient (12 % acrylamide/bis-acylamide of gel percentage) in addition to a balance plate attached to the inner core. Amplicons consisting of U1-U2 rDNA fragments from each patient sample, (30 µL (15 µL PCR product and 15 µL 2× gel loading dye)) were loaded appropriately, in addition to a 28*S* standard ladder (28 µL (14 µL PCR products of 7 different fungal species and 14 µL 2× gel loading dye)) were electrophoresed at 70 V constant (Basic PowerPac™, BIO-RAD) for 17 h, stained with SYBR Green nucleic acid stain I (Invitrogen™), incubated for 30 min at 75 rpm, (Edmund Bühler, KM-2) and de-stained briefly with dH_2O, before being finally visualised under UV light (BIO-RAD Gel Doc 2000 system and Quantity One software (v. 4.6.6)). Hard copies of the fungal community DGGE profiles in the cross-sectional patient cohort were generated as previously described.

2.3.8 Bacterial and fungal DGGE profile capture and analysis

Both bacterial and fungal DGGE profiles generated were first captured in the Quantity One software (v. 4.6.6) program, cropped appropriately and then copied. These copied DGGE profiles were then exported as TIFF (tagged image file format) picture files at 276 dots per inch (DPI) resolution and subsequently imported into TotalLab's Phoretix 1D (TotalLab Ltd.) via a new experimentation file being created. All DGGE profiles imported were subsequently converted into the inverted version of the DGGE profiles, thus measurements were all subsequently inverted. Processing of both the bacterial and fungal DGGE community profiles firstly involved the creation of lanes and appropriate framing before suitable adjustment to compensate for inter-gel variance effects such as lane bending. Once these changes were implemented and accepted, background detection was removed from the DGGE profiles using the rolling ball software algorithm (100 pixel radius) in Phoretix 1D. Detection of bands in gels was performed using the automatic detection algorithm, before bands and gel artefacts were either removed or added manually by eye; band width was also assessed and adjusted manually by eye.

Inter-gel alignment was achieved by executing both 16*S* and 28*S* SLs for bacterial and fungal community detection, important in relative front (Rf) calibration function (Tourlomousis et al., 2010). Comprising of 16*S* and 28*S* rRNA gene amplicons derived from pure cultures of selected relevant species, these served as internal markers in which all lanes were aligned relative to. Lane alignment was manually created and adjusted by the assignment of standard Rf values for the most reproducible bands (in particular the 16*S* SL) present in the standard ladders based upon migration distance through gel as these were approximately the same between gels analysed. Both 16*S* and 28*S* standard ladders contained > 5 reference bands throughout entire patient cohort analysis and for inter-gel alignment this has been suggested for accurate interpolation of multiple gels (Tourlomousis et al., 2010). The Rf lines created were then manipulated to correct for common inter-gel effects such as 'smiling' and 'leaning'. Once accomplished, the current Phoretix 1D experiment was saved and closed.

Using TotalLab's Phoretix 1D Pro program, a database was created in which all of the previously analysed DGGE profiles in Phoretix 1D were subsequently added to this database. Alignment of all gels was first validated by eye and then using the compare lanes software algorithm tool. Once alignment of gels was visually accepted, all gels and their respective standard ladders were matched using the match gel function in Phoretix 1D Pro. Following this, an automatic DGGE band matrix was then created using the Rf values and relative intensities of all detected bands in the experimental file. Both band presence and relative intensities were exported to *Microsoft*® Excel™ for further statistical and ecological analysis. The exported band presence matrix was directly utilised for the species richness in patient cohort (see below). The relative band intensities were normalised as previously described in section 2.1.6.1 (DGGE profile capture and analysis).

2.3.9 Ecological analysis inferred from DGGE profiling

Examination of the structure of the bacterial communities present in the sputum samples analysed by DGGE was performed. Biodiversity measures such as species richness (*R*) (the number of taxa present), evenness (*J'*) (the proportion contributed to the community by each taxa), and Shannon diversity (*H'*) (a diversity index giving a measure of both species numbers and the evenness of their abundance), were calculated using the exported DGGE band matrix in *Microsoft*® Excel™. Note that fungal biodiversity measures could not be carried out due

to scarce detection in patient cohort (only three fungal taxa in whole cohort). The H' derived from the normalised band values in Excel™ has been previously described in section 2.1.6.2 (Shannon diversity index derivation). This was conducted for the entire patient cohort analysed by DGGE and the bacterial community profiles generated. The J' was also derived once the H' was calculated, executed by the following formula:

$$J' = \frac{H'}{\ln(\sum R)}$$

Where J' is the species evenness derived from the natural logarithm (ln) of the sum of the species richness (R) value per lane divided by the H' of that particular lane.

2.3.9.1 Raup-Crick similarity index

The Raup-Crick similarity index was used to assess if the bacterial communities present in each patient were non-randomly ($P = 0.05 \leq$ or ≥ 0.95) or stochastically ($P = > 0.05$ and < 0.95) assembled using the PAST software program in which the probabilities were calculated by 200 pair-wise Monte Carlo simulations (Hammer and Harper, 2001).

2.3.10 Ordination analyses by Canoco

Matrices derived from the normalisation of the band relative intensities were subjected to ordination analyses by using the Canoco (v. 4.5.1) software package with ordination plots being generated by CanoDraw (v. 4.14). In *Microsoft*® Excel™, normalised band matrices were copied onto the clipboard before being transposed into a data file format suitable for Canoco using the WCanoImp (v. 4.5.2.0) plug-in. Environmental variables such as patient phenotypic data (age, gender, current colomycin and azithromycin therapy, etc.) were assigned either contiguous values (e.g., age, FEV_1% predicted) or a binary system to denote indiscreet variables (positive values = 1; negative values = 0).

Band matrices were firstly analysed by detrended correspondence analysis (DCA) in Canoco to determine 1° axis length, used to ascertain what appropriate ordination analysis to use. If 1° axis length was < 3 SD (standard deviation units of species turnover) a redundancy analysis (RDA; a constrained linear ordination method) was executed as opposed to > 3 SD in which canonical correspondence analysis (CCA (a constrained unimodel ordination method)) was chosen instead. Statistical analysis of both the bacterial community, discreet and indiscreet variables was performed using full model Monte Carlo testing (499

permutations) with significant factors identified if *P*-values were < 0.05 and not significant if *P* = > 0.05. Following analysis, analysed data was then imported into CanoDraw for generation of ordination plots containing samples (patient subject number) and environmental factors. Ambiguity between discreet and indiscreet variables was solved by assigning discreet factors with centroids whereas contiguous variables were represented by an arrow. Patient samples analysed by DGGE, i.e, the normalised band matrices plotted, could be denoted by different shapes and colours in CanoDraw for clarity if deemed necessary. Ordination analyses of the bacterial profiles, derived from the initial DGGE analysis seek to represent the similarity between the bacterial communities from each sample. Profiles that have a similar community structure and composition group more closely together. Constrained unimodel (canonical) analyses show variation between the sample profiles that can be explained by the measured categorical and continuous environmental variables (ter Braak and Šmilauer, 2002).

2.4 Molecular fingerprinting and metagenomic analysis in a chronic obstructive pulmonary disease cohort

2.4.1 Sampling methodology and ethical considerations within patient cohort

Eleven clinically stable patients (App. 5), comprised of smokers and ex-smokers with GOLD (Global Initiative for Chronic Obstructive Lung Disease) stages II and III of chronic obstructive pulmonary disease (COPD) attended a routine clinic at the Freeman Hospital, Newcastle upon Tyne, to undergo bronchoscopy, in which a bronchoalveloar lavage (BAL) fluid was produced (except in one patient, where bronchial secretion (BS) fluids were obtained instead due to difficulty of BAL fluid extraction). The BAL and BS fluids were then processed for conventional microbiology testing in the Microbiology Department at the Freeman Hospital, Newcastle upon Tyne, under Health Protection Agency protocols (SOP in App. 4), where the remaining aliquots of clinical specimen fluids were stored at -80 °C prior to microbial gDNA extraction. Ethical approval for this study was granted by Northumbria University's School of Life Sciences Research Ethics Committee and Newcastle upon Tyne Hospitals *NHS* Foundation Trust.

2.4.2 DNA extraction from bronchoalveolar lavage and bronchial secretion fluid clinical samples

Microbial gDNA extraction from BAL and BS fluid samples was achieved using an UltraClean® Microbial DNA Isolation Kit (MO BIO Laboratories, Inc.) in a Microflow Peroxide Advanced Bio Safety Cabinet (Class I) at the Freeman Hospital, Microbiology Department, Newcastle upon Tyne with amendments to the kit protocol. All fluid samples (< 5 mL in volume) contained in a 25 mL plastic universal were thawed thoroughly and then vortexed (Vortex-Genie2™) rigorously with addition of an equal volume of sputolysin (added only to slightly purulent fluid samples to aid in breakage of any mucus plugs present and enabling passage of the supernatant through the 0.22 μm filter (in later stages of this procedure)). Both the BAL and BS fluid samples were then centrifuged at 3000 × *g* for 10 min at room temperature (CENTAUR, P20075 rota) before the supernatant was decanted carefully and the cellular pellet resuspended in 300 μL of MicroBead Solution within the 25 mL universal tube. This was gently vortexed and 500 μL of this mix was then transferred to a MicroBead Tube in which 50 μL of MD1 solution was added.

The MicroBead Tubes were then secured horizontally using the MO BIO Vortex Adaptor tube holder and vortexed at maximum speed for 10 min before being spun down at 10 000 × *g* (SIGMA® 12094 rotor) at room temperature for 1 min in a microcentrifuge (SIGMA®). The supernatant was then transferred to a clean, sterile 2 mL Collection Tube with ~ 300-350 μL of supernatant present. One hundred μL of Solution MD2 was then added to the supernatant and vortexed for 5 s and then incubated at 4 °C for 5 min. Following this incubation step, the 2 mL Collection Tubes were then centrifuged at 10 000 × *g* for 1 min at room temperature. A 2 mL syringe (BD) was attached to a filter (PALL Gelman Laboratory, Acrodisc® Syringe Filter, 0.2 μm Supor® membrane, non-pyrogenic) and the syringe plunger removed and placed to one side. The entire supernatant medium from the 2 mL Collection Tube was then transferred to the empty syringe vial and filter-sterilised, via re-attachment of the syringe plunger into the syringe vial, by pushing the supernatant through the 0.2 μm filter into another clean, sterile 2 mL Collection Tube. As previously described, this filter-sterilisation process was an extra step introduced into the gDNA extraction procedure to circumvent possible exposure to any tubercular agents that may have been present in the clinical sample. Less than 300 μL of supernatant volume should have been dispensed from the filter, although this varied due to the amount of BAL and BS fluids

originating from the 25 mL universal tube. Syringe and filter apparatus were discarded after use and only used once.

Nine hundred µL of shaken and mixed Solution MD3 was then added to the filtered supernatant, vortexed for 5 s, before 750 µL of this mixture was added to a Spin Filter before being initially centrifuged at 10 000 × *g* for 1 min at room temperature. Any flow through liquid was discarded and the remaining supernatant was added to the Spin Filter and centrifuged again at 10 000 × *g* for 1 min at room temperature. All flow through liquid was discarded. Three hundred µL of Solution MD4 was then added to the Spin Filter before being centrifuged at 10 000 × *g* for 1 min at room temperature. Again, any flow through liquid was discarded and the Spin Filter was centrifuged at 10 000 × *g* for 1 min at room temperature. The spin filter basket was removed and the Spin Filter was placed into a new 2 mL Collection Tube in which 50 µL of Solution MD5 was pipetted directly into the centre of the white filter membrane. A final centrifugation at 10 000 × *g* at room temperature for 1 min was required to release the gDNA (50 µL in volume) from the filter membrane. Following this, the Spin Filter itself was discarded and the eluted gDNA from the BAL and BS samples was stored at -80 °C prior to downstream processing.

2.4.3 'Semi-nested' bacterial community detection PCR in BAL and BS fluid samples

As previously described in the nCFBR materials and methods sections, the bacterial community in the eleven COPD patients enrolled was amplified using *Ex Taq*™ HS pol (TAKARA BIO INC.). Primers V3F-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′) and V3R (5′- ATT ACC GCG GCT GCT GG-3′) targeted the V3 hypervariable region of the 16*S* rRNA gene resulting in the enzymatic amplification of V3 rDNA amplicons using extracted gDNA as template as previously shown (Muyzer et al., 1993). As before, the forward primer V3F-GC had a 40-bp GC-rich nucleotide sequence attached to its 5′ end enabling greater detection of variances in bacterial gDNA within the V3 rDNA amplicons and PCR fragment stability when analysed by DGGE (Sheffield et al., 1989).

An automatic HS and touchdown PCR protocol was performed on a ***Mastercycler gradient*** (Eppendorf) with the PCR reagents per reaction added as follows: 0.5 µM primers (both forward (V3F-GC) and reverse (V3R)), 1× Ex Taq Buffer (TAKARA BIO INC.), 0.2

mM each dNTP (TAKARA BIO INC.), 1 mM $MgCl_2$, 10 µg/µL BSA (Promega), 1.25 U *Ex Taq*™ HS pol and 1 µL of gDNA template made up to a total volume of 50 µL with sterile 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). Positive (extracted gDNA from a pure culture of *H. influenzae*, NCTC no. 11931) and negative controls were also set up to determine the correct size of the V3 rDNA fragments and to rule out any environmental DNA contamination. Samples were incubated for 5 min at 97 °C releasing antibody bound polymerase *Ex Taq*™ HS, then at 96 °C for 1 min, 65 °C for 1 min, with a reduction of 0.5 °C per cycle until touchdown at 55 °C. Primer extension lasted for 30 s at 72 °C and this was repeated for 20 cycles. An additional fifteen cycles was carried out at 94 °C for 1 min, 55 °C for 1 min, 72 °C for 3 min and a final extension time of 72 °C for 30 min. Following this completed reaction, the V3 rDNA amplicons were then used as DNA template for a second round of bacterial community amplification with exactly the same concentrations and conditions outlined above (a 'semi-nested' approach). As previously described, implementation of both a touchdown protocol and a longer final extension time was executed to circumvent spurious PCR product and double band formation bias on DGGE analyses (Don et al., 1991, Janse et al., 2004). These implementations were also executed on the consecutive round of PCR when the V3 rDNA fragments were utilised as template.

2.4.4 'Semi-nested' fungal community detection PCR in BAL and BS fluid samples

As in the cross-sectional nCFBR patient cohorts, community fungal PCR on a ***Mastercycler gradient*** (Eppendorf) was also performed in addition to universal bacterial community detection. PCR primers specific for the amplification of variable regions within the 28*S* rRNA gene ubiquitous in the fungal genome were utilized as shown previously (Sandhu et al., 1995). PCR using the U1F (5′-GTG AAA TTG TTG AAA GGG AA-3′) and U2R-GC (5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG-3′) primer sets were executed, in addition to *Ex Taq*™ HS pol (TAKARA BIO INC.) to enzymatically amplify small segments of 28*S* rDNA (U1-U2 rDNA fragments).

PCR reagents for this reaction were added as follows: 0.5 µM primers (both forward (U1F) and reverse (U2R-GC)), 1× Ex Taq Buffer (TAKARA BIO INC.), 0.2 mM each dNTP (TAKARA BIO INC.), 1 mM $MgCl_2$, 10 µg/µL BSA (Promega), 1.25 U *Ex Taq*™ HS and 1 µL of gDNA template made up to a total volume of 50 µL with sterile 18.2 MΩ H_2O

(MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter). An automatic HS and touchdown PCR protocol was utilised in which the conditions were an initial denaturation step of 97 °C for 5 min, 94 °C for 1 min, 60 °C for 1 min with a reduction of 1.0 °C per cycle reaching touchdown at 50 °C and 72 °C for 1 min repeated for 10 cycles. An additional 25 cycles were performed at 94 °C for 3 min, 50 °C for 1 min, and 72 °C for 1 min. As previously introduced, a final extension time at 72 °C for 30 min was implemented in addition to the touchdown protocol ((Don et al., 1991, Janse et al., 2004). Following this completed reaction, the U1-U2 rDNA amplicons were then used as DNA template for a second round of fungal community amplification with exactly the same concentrations and conditions outlined above (a 'semi-nested' approach). Both positive and negative controls were executed for confirmation of the correct size of the fungal U1-U2 rDNA amplicons (using extracted gDNA from a pure culture of *A. fumigatus*) and to rule out any environmental sources of contamination.

2.4.5 Agarose gel electrophoresis of both V3 and U1-U2 rDNA amplicons from BAL and BS fluid samples

Amplicons generated from bacterial and fungal community PCR from BAL and BS fluid samples were validated for positive amplification of their respective 16*S* and 28*S* rRNA gene segments by running out PCR fragments on an agarose gel (1 % ($^{w}/_{v}$) and electrophoresing at 120 mA constant for 20 min (BIO-RAD, Basic PowerPac™). The agarose gel was stained with SYBR® Safe DNA gel stain (10 000× concentrate in DMSO) (Invitrogen™) prior to use for visualisation under UV light. Five µL of PCR product, 2 µL 6× BPB, and 5 µL 18.2 MΩ H_2O (MILLIPORE, Direct-Q™ Ultra Pure Water Systems, MILLIPAK® 0.22 µm filter) were mixed by trituration before a final volume of 10 µL was loaded into the appropriate gel well, in addition to 5 µL of Hyperladder 1 (BIOLINE). Using a BIO-RAD Gel Doc 2000 system and Quantity One software (v. 4.6.6) comparison of the molecular base pair markers in Hyperladder I (BIOLINE), in addition to the positive control, confirmed the correct sizes of both types of amplicons; i.e., V3 and U1-U2 rDNA fragments (233 bp and 300 bp in size respectively). Hard copies of the electropherograms were generated using a Mitsubishi Video copy processor (model P91 attached to the Gel Doc 2000 system).

2.4.6 Molecular fingerprinting of the bacterial and fungal community within BAL and BS fluid samples

2.4.6.1 DGGE of V3 rDNA amplicons from BAL and BS fluid samples

Bacterial molecular fingerprinting within the BAL and BS clinical samples was carried out using DGGE as previously described on a DCode™ Universal Mutation Detection System (BIO-RAD) on a 32.5-60 % denaturing gradient (12 % acrylamide/bis-acrylamide of gel percentage). Amplicons from all 11 patients in addition to a 16*S* SL consisting both of V3 rDNA fragments were loaded appropriately into wells; 30 µL (15 µL PCR product and 15 µL 2× gel loading dye) and 36 µL (18 µL PCR products of 9 different bacterial species (see above) and 18 µL 2× gel loading dye) respectively. PCR fragments were electrophoresed for 4.5 h at 200 V constant (Basic PowerPac™, BIO-RAD), stained with SYBR Green nucleic acid stain I (Invitrogen™) and incubated for 30 min at 75 rpm (Edmund Bühler, KM-2), de-stained briefly with dH_2O, before being finally visualised under UV light (BIO-RAD Gel Doc 2000 system and Quantity One software (v. 4.6.6)). A hard copy of the bacterial community DGGE profile in this particular patient cohort was also generated (Mitsubishi Video copy processor (model P91) attached to the Gel Doc 2000 system).

2.4.6.2 DGGE of U1-U2 rDNA amplicons from BAL and BS fluid samples

Fungal community analysis of COPD cohort was performed using the DCode™ Universal Mutation Detection System (BIO-RAD) on a 40-60 % denaturing gradient (12 % acrylamide/bis-acylamide of gel percentage). As before, all eleven patient amplicons (30 µL (15 µL PCR product and 15 µL 2× gel loading dye)) were loaded appropriately, in addition to a 28S SL (28 µL (14 µL PCR products of 7 different fungal species (see above) and 14 µL 2× gel loading dye)). Electrophoresis of U1-U2 rDNA amplicons was executed at 70 V constant (Basic PowerPac™, BIO-RAD) for 17 h, stained with SYBR Green nucleic acid stain I (Invitrogen™) and incubated for 30 min at 75 rpm, (Edmund Bühler, KM-2) de-stained briefly with dH_2O, before being finally visualised under UV light (BIO-RAD Gel Doc 2000 system and Quantity One software (v. 4.6.6)). A hard copy of the fungal community DGGE profile in the COPD patient cohort was also generated (Mitsubishi Video copy processor (model P91) attached to the Gel Doc 2000 system).

2.4.7 Metagenomic analysis of BAL and BS fluid samples

Extraction of gDNA from both BAL and BS fluid samples yielded 50 µL of eluted gDNA, 20 µL of which was sent to Research and Testing Laboratories (RTL) LLC. (Lubbock, Texas, U.S.) for 454-pyrosequencing generating the metagenomic data within patient cohort. The bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTEFAP) method targeted the V3-V5 hypervariable regions of the 16*S* rRNA gene creating amplicon libraries (Dowd et al., 2008). Primer sets corresponded to 341F (5′-CC TAC GGG AGG CAG CAG-3′) and 907R (5′-CC GTC AAT TCC TTT GAG TTT-3′) co-ordinates of the reference *E. coli* 16*S* rRNA gene in addition to sample nucleotide bar codes. Roche A & B primers were then implemented to perform pyrosequencing by established methods (Dowd et al., 2008). Resulting 16*S* pyroamplicon sequences were then pre-processed and aligned using the SILVA reference alignment database, before being trimmed and eliminated for chimeras as previously described (Costello et al., 2009) in the open-source, platform-independent, community-supported software program mother (http://www.mothur.org) (Schloss et al., 2009). The processed 16*S* pyroamplicon reads were then subsequently analysed and assigned OTUs with a 3 % cutoff with further analysis of the metagenomic data following the Costello Stool Analysis example (http://www.mothur.org/wiki/Costello_stool_analysis).

2.4.8 Statistical and ecological analysis of COPD patient cohort

From both the PCR-DGGE and metagenomic profiles generated, these were analysed using Canoco for Windows (v.4.5.1) with the ordination plots displayed in CanoDraw (v. 4.14). Band and metagenomic matrices data was transposed into a suitable format for Canoco for Windows using WCanoImp (v. 4.5.2.0) plug-in and ordination plots and subsequent analysis was performed as previously described in section 2.3.10 (Ordination analyses by Canoco).

Chapter Three: DNA polymerase enzymes and microbial diversity

3.1 Abstract

Molecular fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) enable the analysis of microbial communities in a variety of environments. However, a 'gold standard' DNA polymerase (pol) enzyme used to determine the microbial diversity in any given sample type is yet to be established. Reviewing the current literature, five DNA polymerase enzymes, Platinum® *Pfx* (Invitrogen™), *TaKaRa Ex Taq*™ Hot Start (TAKARA BIO INC.), AmpliTaq® (Applied Biosystems™), GoTaq® Hot Start Polymerase (Promega), and *Taq* (New England BioLabs®, Inc.) have all been widely utilised to study microbial diversity from a variety of different environments. These five DNA pol enzymes were assessed to determine the microbial diversity generated in sandy soil taken from dunes on Longsands Beach, Tynemouth, North East England. Using DNA extraction to obtain genomic DNA (gDNA) from sandy soil, the variable V3 region of the 16*S* rRNA gene was amplified with the five DNA pol using PCR conditions set out by the manufacturer's recommendations for each enzyme. The PCR products from all five DNA pol reactions were compared by DGGE and multivariate analysis to determine the similarity of the profiles. Data strongly suggests that Platinum® *Pfx* and *TaKaRa Ex Taq*™ Hot Start detected greater microbial diversity present within sandy soil than the other DNA pol enzymes employed.

3.2 Background

Since the origins of life on Earth, polynucleotide pols exhibited the first signs of enzymatic activity early in the course of evolution; if absent, accurate and efficient genome replication would not have occurred, thus preventing the pre-requisites of evolution itself (Steitz, 1999). Evidently, accurate processing of genetic information in all organisms from parent to offspring is an absolutely essential process if the preservation and survival of all species is to continue; however, a critical step in species continuity is the tolerances in base-pair mutation rates occurring over time, but providing life with an evolutionary selective advantage over other species (Perler et al., 1996). This preservation of the Watson-Crick structure in DNA during replication and repair is performed by DNA pol enzymes (Beard and

Wilson, 2003), a ubiquitous family of enzymes that are composed of functionally distinct domains and subdomains (Steitz, 1999). Characteristics of the DNA pol enzymes are often described as having a broad structural homology to that of the right hand, with "thumb", "palm" and "fingers" sub-domains, all a common structural theme (Kohlstaedt et al., 1992). These enzymes can be further divided into several distinct families deduced from the structural homologies displayed from crystallographic studies and amino acid sequences in which the DNA pol families A, B, C, D, X and Y have all been characterised (Bebenek and Kunkel, 2004, Delarue et al., 1990, Joyce and Steitz, 1994). The best known and the most intensively studied is the DNA pols from the A family which include DNA pol I and Klenow fragments (a truncated version of this enzyme, which includes the C-terminal portion, containing the 3′ → 5′-exonuclease and pol domains) isolated from *Escherichia coli* and *Bacillus* DNA pol I in which the crystal structures of the above have been previously published (Ollis et al., 1985, Beese et al., 1993, Kiefer et al., 1998). Different DNA pol families exhibit general functions: (i), families B, C, and D and their respective DNA pols appear to be involved in chromosomal replication, (ii), A and B families exclusively promote the replication of DNA in viruses and bacteriophages, and (iii), DNA repair mechanisms are incorporated in all six families (Pavlov et al., 2004).

Structurally homologous to all DNA pol families is the palm sub-domain — the X family is unusual in that the DNA pol enzymes are described as being "left handed" and thus not homologous with the other families above (Steitz et al., 1994, Beard and Wilson, 2000) — whereas across all DNA pol families, the finger and thumb domains differ in structure (Brautigam and Steitz, 1998). The palm sub-domain provides the catalytic action necessary for the addition of nucleotides via three catalytic residues that co-ordinate with two essential divalent metal ions that assist in the nucleotidyl transferase reaction (Steitz et al., 1994). The acquisition of two magnesium (Mg^{2+}) cations is critical in the accurate synthesis of DNA, however, manganese (Mn^{2+}) cations can be implemented as opposed to Mg^{2+} ions resulting in an increase in synthesising error rate during DNA replication but enabling numerous DNA pols to use RNA template as an alternative to DNA (Pavlov et al., 2004). Despite all of these differences in their tertiary structures, DNA pols all operate and synthesise new nucleotides via a standard catalysed reaction in which the addition of a nucleotide is added to the 3′-end of a DNA primer.

In the polymerase chain reaction (PCR) thermostable DNA pols are a necessity if one is to effectively select for and produce many copies of the gene of interest in addition to the subsequent isolation, characterisation and DNA template required for sequencing. In a sequence of events that led to the development of the polymerase chain reaction (PCR) (Saiki et al., 1985), the discovery of the thermophile (a micro-organism which proliferates optimally at < 80 °C) *Thermus aquaticus* and the isolation of its respective DNA pol I enzyme (Chien et al., 1976, Kaledin et al., 1980) has revolutionised thermocycling by enabling automation via the facilitation of thermostable *Taq* pol in PCR. Since the 1980s, the number of commercially available thermostable enzymes for automated thermocycling has risen rapidly, leading investigators to address specific questions such as which DNA pol is suitable for the investigation to be performed and will a 'gold standard' of DNA pol ever come into commercial viability?

Thermostable DNA pols are derived from both thermophilic and hyperthermophilic eubacterial species; prior to this, many of the inherent properties of DNA pol enzymes were studied from the isolation and characterisation of said enzymes from mesophiles such as *E. coli* (Perler et al., 1996). In addition to exhibiting common structural themes derived from crystallographic studies, DNA pol enzymes also exhibit variances in their thermostability, extension rate, processivity and fidelity; all essential properties of thermostable DNA polymerases. Efficient replication of DNA in automated thermocycling must utilise thermostable DNA pol enzymes. Indeed, thermostability of all DNA pols is a critical property as free template DNA must be liberated during the DNA melting step, each cycle involving temperatures at ~ 95 °C, a necessity if new strands of double-stranded DNA (dsDNA) are to be synthesised in amplification procedures. The specific extension rate of a DNA pol is expressed as the number of dNTPs (deoxyribonucleoside triphosphates) polymerised per second per molecule of DNA polymerase, whereas processivity is characterised by the mean number of bases affixed by a DNA pol in a single binding episode during DNA synthesis (Pavlov et al., 2004). As in the other essential preceding properties, DNA pol enzymes also display an intrinsic property termed fidelity, defined as the comparison of the ability of a pol enzyme to insert a correct nucleotide versus an incorrect base (Beard and Wilson, 2003), this efficiency can vary 10^7-fold (Beard et al., 2002, Kunkel, 2004). Inefficient DNA polymerases are naturally occurring enzymes that generally display poor correct nucleotide insertion efficiency, i.e., low fidelity, whereas more efficient DNA pols exhibit a higher fidelity, an

increased capacity for correct base insertion during DNA replication and other mechanisms (Beard and Wilson, 2003).

As previously stated, the inherent microbial diversity within Earth's biosphere is vast. Due to the limitations in using culture-dependent techniques and the 'great plate count anomaly' a paradigm was created in that an incomprehensible amount of bacteria are uncultivable and are therefore alluded to us (Amman et al., 1995, Rappé and Giovannoni, 2003, Staley and Konopka, 1985). However, in the last two decades culture-independent methodologies encompassing molecular fingerprinting techniques such as DGGE have emerged to provide investigators with powerful tools for discerning the many microbial communities that comprise the biosphere. Microbial communities have been identified and characterised from an extensive number of dissimilar natural habitats, not only around us, but also within us (Postec et al., 2005, Fujimoto et al., 2003, Vitali et al., 2007, Brakstad et al., 2008, Edenborn and Sexstone, 2007, van de Wiele et al., 2004).

In terms of the other molecular fingerprinting methods briefly elaborated on, DGGE is one of the most established culture-independent techniques used in molecular microbial ecology studies (Muyzer and Smalla, 1998) as for most laboratories the initial implementation and cost-effectiveness of next generation sequencing (NGS) platforms is too high. Operating on principles and modifications since 1983 when it was first introduced (Fischer and Lerman, 1983); small PCR fragments (~ 200-700 base pairs (bp)) are amplified from variable regions within the 16*S* rRNA gene representing members from the bacterial community. Amplification of these variable regions (e.g., the V3 region yielding V3 rDNA fragments) is usually performed by universal PCR primers resulting in amplicons of approximately the same length, thus, PCR products analysed by DGGE cannot be separated on traditional agarose or non-denaturing acrylamide gels. The bacterial community produced from the pool of V3 rDNA amplicons from extracted total gDNA in either environmental or clinical samples are separated out on a linear chemical denaturing gradient (urea and formamide) embedded within an acrylamide gel matrix according to genome fragment base-composition, i.e., guanine (G) and cytosine (C) content (Hovig et al., 1991) and a decrease in the electrophoretic mobility of the partially melted dsDNA molecules (Muyzer, 1999). Differential migration of these heterogeneous PCR amplicons containing gDNA sequence variation is achieved by the amount of denaturant in the gradient and the dissociation of the

two DNA strands containing differences in the number of hydrogen (H) bonds between the complementary nucleotides binding the DNA strands together; i.e., three H bonds bind G and C, as opposed to adenine (A) and thymine (T) which only contain two H bonds (Nakatsu, 2007). Thus, PCR fragments containing a greater amount of GC-rich amplicons will take longer to denature and dissociate migrating towards the bottom of the gradient in which the partial melting point of these GC-rich DNA fragments is reached, whereas AT-rich DNA sequences will migrate towards the top of the denaturant gradient. Once this partial melting temperature has been achieved, the DNA fragments can no longer migrate through the denaturing gradient and are immobilised because of the transition of the DNA macromolecule from a helical to partially melted structure, thus dsDNA sequence variation is not only separated out by GC-content, but also variations in the partial melting temperatures thus affecting dissimilar immobilisation points within the linear denaturing gradient of the PCR amplicons analysed (Muyzer and Smalla, 1998). Further to this, the attachment of a GC-clamp (a GC-rich nucleotide sequence) to the 5′-end of one of the universal primers can facilitate greater detection of sequence variants within the community being analysed as this increases PCR fragment stability as it migrates through the denaturing gradient, increasing the melting temperature, thus preventing complete dissociation of the dsDNA into single-stranded DNA (ssDNA) (Muyzer and Smalla, 1998). Attachment of a GC-clamp in PCR-DGGE mediated studies in microbial ecology studies under optimum running conditions in DGGE can result in the detection of ~ 100 % of sequence variants within the microbial community in DNA fragments ≤ 500 bp in size (Sheffield et al., 1989, Myers et al., 1985). Thus DGGE can represent a global 'snapshot' of the microbial population structure and diversity analysed by said technique (Nakatsu, 2007) from when it was first introduced to study bacterial communities in soil (Muyzer et al., 1993).

In PCR-DGGE mediated studies of environmental samples and indeed human clinical samples, a variety of thermostable DNA polymerases are utilised in order to ascertain the microbial communities present. In this study, five DNA pols were assessed to analyse the bacterial communities in sandy soil obtained from dunes on Longsands Beach, Tynemouth, North East England. These were, AmpliTaq® (Applied Biosystems™), GoTaq® Hot Start Polymerase (Promega), Platinum® *Pfx* (Invitrogen™), *TaKaRa Ex Taq*™ Hot Start (TAKARA BIO INC.), and *Taq* (New England BioLabs®, Inc.). All of the thermostable DNA polymerases used in this study were originally isolated from strains of *T. aquaticus*

expressing modified versions of the DNA polymerase gene which were then inserted and expressed in strains of *E. coli* (Lawyer et al., 1989) thus belonging to the A polymerase family (Kim; Eom refs.). The exception being Platinum® *Pfx* — cloned and purified from the archaeon *Pyrococcus* spp. strain KOD1 a member of the B polymerase family (Westfall et al., 1999, Takagi et al., 1997, Nishioka et al., 2001). Comparison of the DNA polymerase enzymes assessed in this study are summarised below in table 3.1.

Table 3.1: Comparison of DNA pols utilised to determine microbial diversity in sandy soil samples.

DNA polymerase	DNA polymerase family group	Supplier	Average error rate reported‡
Taq	A	New England BioLabs® Inc.	285×10^{-6} bp
GoTaq® Hot Start	A	Promega	~ 8.0×10^{-6} bp†
AmpliTaq®	A	Applied Biosystems™	Not available
Ex Taq™ Hot Start	A	TAKARA BIO INC.	8.7×10^{-6} bp
Platinum® *Pfx*	B	Invitrogen™	1.58×10^{-6} bp

‡ Determined by the Cline method (Cline et al., 1996).

† Was not determined, error rate is based on older versions of *Taq* DNA pol that Promega used to distribute.

Different DNA pols from the above were assessed in order to select the most appropriate DNA polymerase enzyme prior to study of clinical samples involving sputum and bronchoalveolar lavage fluid from multiple patient cohorts with chronic respiratory tract diseases. Sandy soil obtained from dunes was utilised in this assessment for several reasons: (i), no ethical approval was needed prior to initial sample excavation, (ii), the microbial communities inhabiting the soil microenvironment are genetically diverse, with estimates ranging from 467 to 500,000 species of bacteria present (Curtis et al., 2002, Hughes et al., 2001, Torvisk et al., 1998, Dykhuizen, 1998) — thus if the DNA pol enzymes assessed were to effectively amplify DNA template producing V3 rDNA amplicons this would be detected by DGGE strongly indicating the dominant microbial taxa present in sandy soil — and (iii),

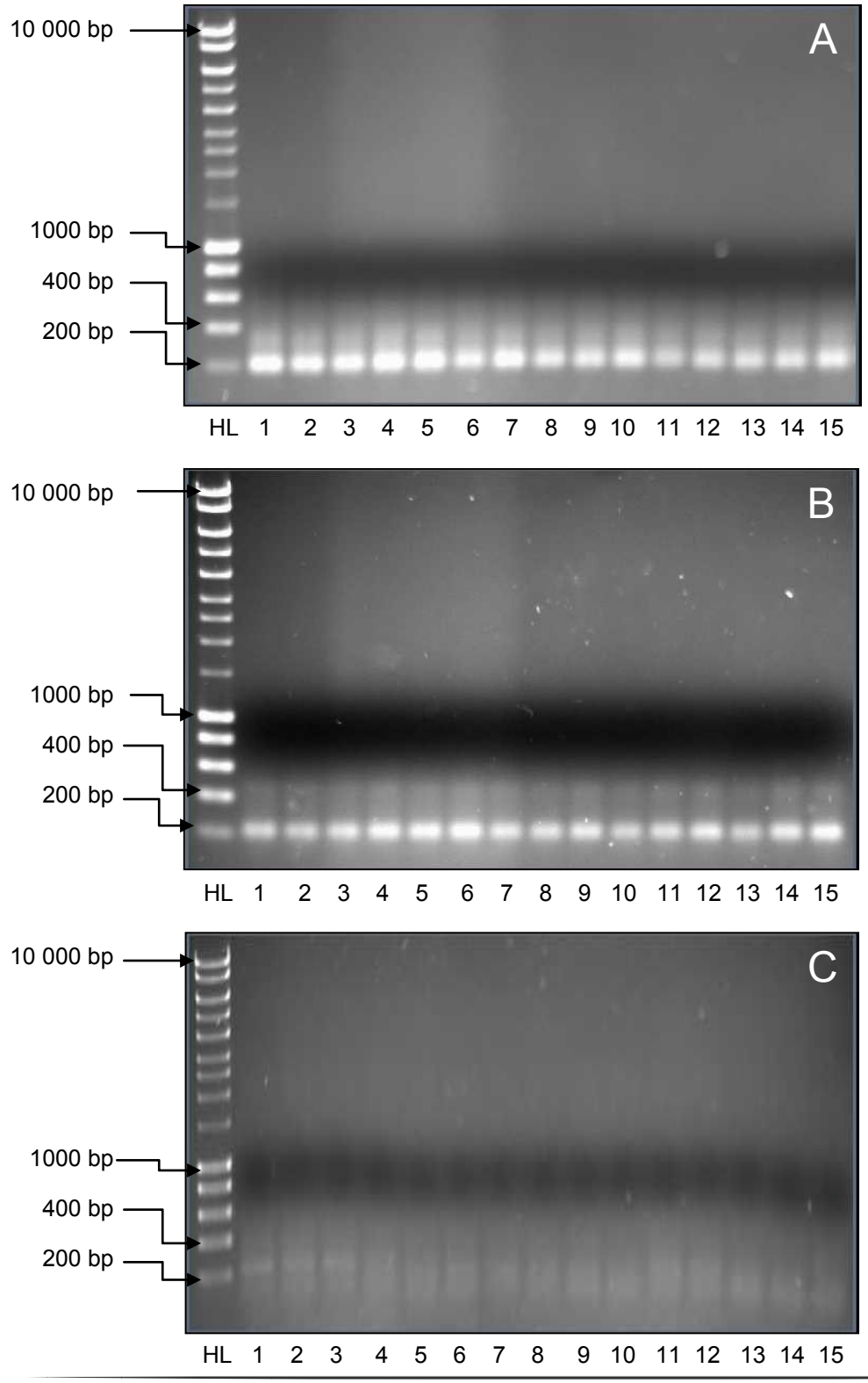
humic acids consisting of complex polyphenolic compounds produced from the decomposition of organic matter within soil sediments can strongly inhibit PCR (Tsai and Olsen, 1992b, Tsai and Olsen, 1992a), sandy soil contains less of these interfering humic substances due to a relative lack of organic matter content as opposed to agricultural soils. From here, once the appropriate DGGE profiles were generated, statistical methods were executed to ascertain if there was a difference within the community generated from the pooled V3 rDNA fragments amplified in each sandy soil sample analysed by each DNA pol enzyme employed.

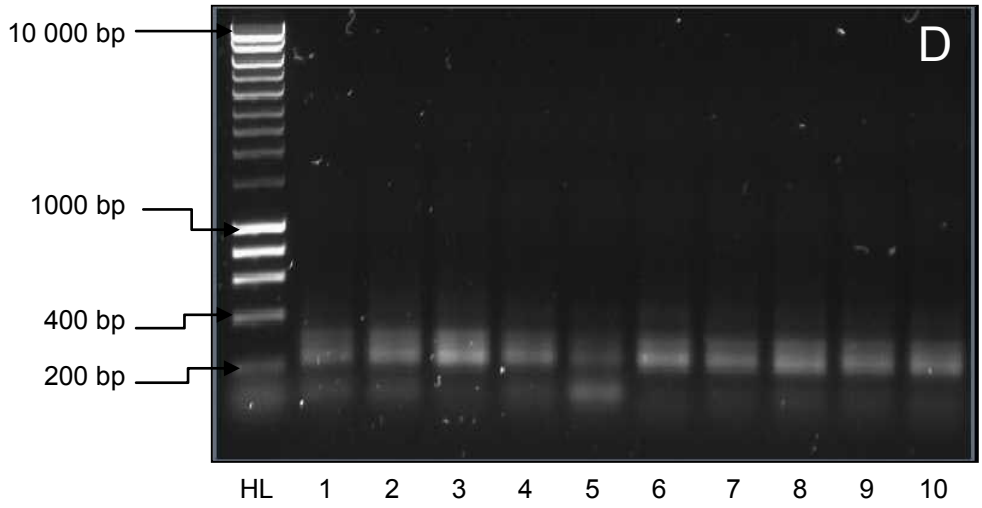
3.3 Results

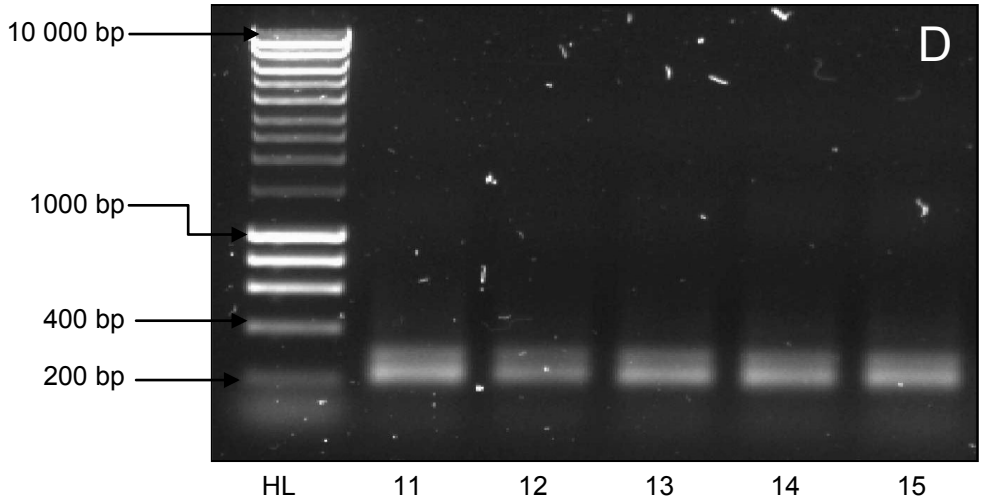
3.3.1 Optimisation of PCR amplifications

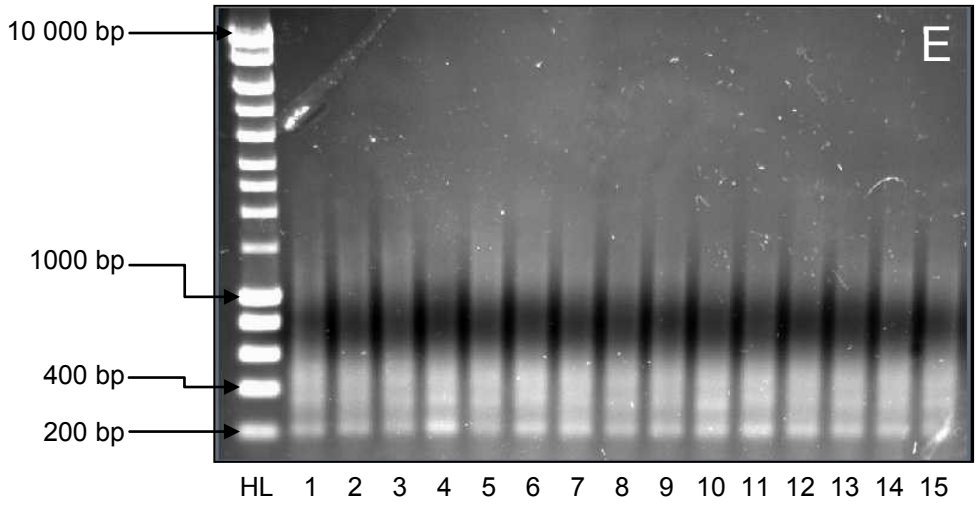
As previously described in Chapter Two, steps were taken to minimise artefacts, spurious and heteroduplex PCR products (Don et al., 1991, Janse et al., 2004); relief of sample inhibitors was also implemented (Kreader, 1996). In terms of optimisation of each DNA pol assessed, both *Ex Taq*™ HS and GoTaq® HS followed the protocol closely outlined as previously described (Muyzer et al., 1993) producing the amplifications in figure 3.1A and B. The only differences between the *Ex Taq*™ HS and GoTaq® HS PCR experiments were buffer, dNTP, and $MgCl_2$ concentrations per reaction. Applied Biosystems™ AmpliTaq® was optimised using an $MgCl_2$ concentration gradient set-up experiment (0.5-2 mM $MgCl_2$), optimum $MgCl_2$ concentration was 1 mM. However, despite repeated attempts at adjustment of GeneAmp® Buffer and AmpliTaq® DNA pol volumes in multiple reactions, V3 rDNA amplification was still poor (figure 3.1C). *Taq* (New England BioLabs® Inc.) DNA pol was optimised by slight adjustment to the annealing temperature using a temperature gradient (ranging from 53-63 °C, optimum annealing temperature was at 55.8 °C) and addition of 5 U *Taq* pol instead of 2.5 U. *Pfx* DNA pol was optimised by the addition of *Pfx* Enhancer Solution (Invitrogen™) at 0.5 × concentration as opposed to 1 × concentration. Final amplification of V3 rDNA fragments using both *Taq* and *Pfx* are shown in figures 3.1D and E respectively.

Fig. 3.1A-E: Gel images showing final PCR amplifications of V3 rDNA fragments by each DNA pol technical replicate in sandy soil samples 1-5. A, *Ex Taq*™ HS amplification; B, GoTaq® HS amplification; C, AmpliTaq® amplification; D, *Taq* amplification; E, *Pfx* amplification. HL, Hyperladder I (10 000-200 bp) in which four molecular markers are annotated for clarity. Lane numbers: 1-3, SS1R V3 rDNA amplicons; 4-6, SS2R V3 rDNA amplicons; 7-9, SS3R V3 rDNA amplicons; 10-12, SS4R V3 rDNA amplicons; 13-15, SS5R V3 rDNA amplicons.




10 000 bp
1000 bp
400 bp
200 bp
D
HL 1 2 3 4 5 6 7 8 9 10


10 000 bp
1000 bp
400 bp
200 bp
D
HL 11 12 13 14 15


10 000 bp
1000 bp
400 bp
200 bp
E
HL 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

3.3.2 Ecological analysis from DGGE profiles generated

Each commercially available DNA polymerase enzyme was assessed in all five sandy soil samples by employment of technical replicates to ensure PCR bias was countered for (within a certain degree) when the appropriate statistical analysis was performed (below). Ecological analysis of the bacterial community generated in each DGGE profile varied. Isolated distinct bands migrating through the denaturing gradient were treated as a single bacterial taxon from which the mean species richness and diversity indices (i.e., Shannon diversity index scores (*H′*)) was calculated from the technical replicates of the five different DNA pol enzymes in addition to the average number of bands detected (summarised in table 3.2).

Across sample numbers 1-5, both high-fidelity enzymes *Ex Taq*™ HS and *Pfx* generated the greatest mean number of bands in each DGGE profile analysed and highest mean *H′* scores (the only exception being in SS3R where the mean *H′* score (2.30) of *Ex Taq*™ HS was equivalent to GoTaq® HS). Applied Biosystems™ AmpliTaq® detected the lowest number of bands (only 1 in SS5R as opposed to Platinum® *Pfx* in which 20.7 bands were detected on average) across all five samples in addition to the lowest mean *H′* scores, again, taking into consideration that no *H′* indices could be calculated for SS5R (each AmpliTaq® replicate only detected one bacterial taxa) and in SS4R as previously mentioned above. Overall bacterial diversity in the five sandy soil sample types is summarised in table 3.3. SS3R yielded the highest detection of bacterial diversity (*H′* range 0.66-3.16 (mean = 2.31)) in addition to also showing the highest band range (2-25 (mean = 13.5)) whereas SS4R displayed the lowest (*H′* range 0.61-3.03 (mean = 1.95)). Both SS3R and SS4R produced a wide range in their *H′* scores, it should be noted however that in two of the AmpliTaq® technical replicates SS4R and SS5R (i.e., all AmpliTaq® replicates) Shannon diversity indices could not be derived as only one bacterial taxa each was detected.

Table 3.2: Comparison of mean number of bands detected and *H′* scores calculated using technical replicates of the five DNA polymerase enzymes assessed.

Sample replicate number	**DNA pol enzyme**[§]	**Mean number of bands detected by each DNA pol**	**Mean *H′* score generated by each DNA pol (*n* = 5)**
SS1R	*Taq*	7.7	1.94
	GoTaq® HS	12	2.29
	AmpliTaq®	4	1.29
	Ex Taq™ HS	18.3	2.83
	Pfx	16	2.65
SS2R	*Taq*	7	1.84
	GoTaq® HS	10.7	2.30
	AmpliTaq®	3.7	1.27
	Ex Taq™ HS	11.7	2.30
	Pfx	12	2.42
SS3R	*Taq*	9.3	2.16
	GoTaq® HS	11.7	2.37
	AmpliTaq®	3.7	1.06
	Ex Taq™ HS	18	2.80
	Pfx	25	3.16
SS4R	*Taq*	8	1.94
	GoTaq® HS	10	2.20
	AmpliTaq®	1.3	0.20
	Ex Taq™ HS	21	3.02
	Pfx	14	2.39
SS5R	*Taq*	11.3	2.36
	GoTaq® HS	9.7	2.16
	AmpliTaq®	1	0[†]
	Ex Taq™ HS	17.7	2.76
	Pfx	20.7	2.87

§ In order of loading on DGGE profile generated.

† Average *H′* score cannot be derived if only one bacterial taxon is detected across all three AmpliTaq® technical replicates.

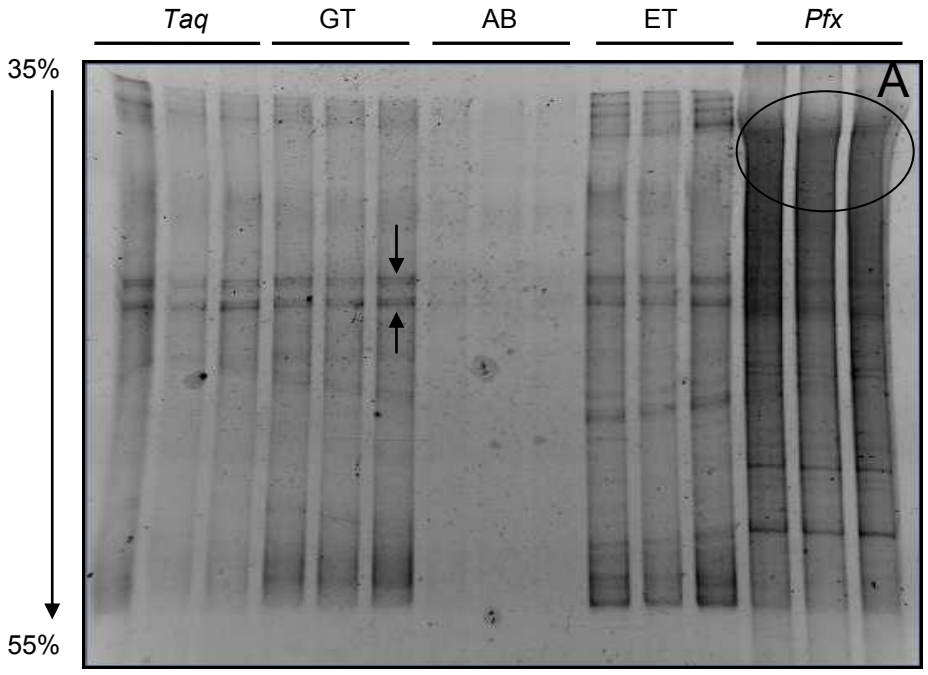
Table 3.3: Comparison of bacterial diversity in the five sandy soil samples analysed by DGGE.

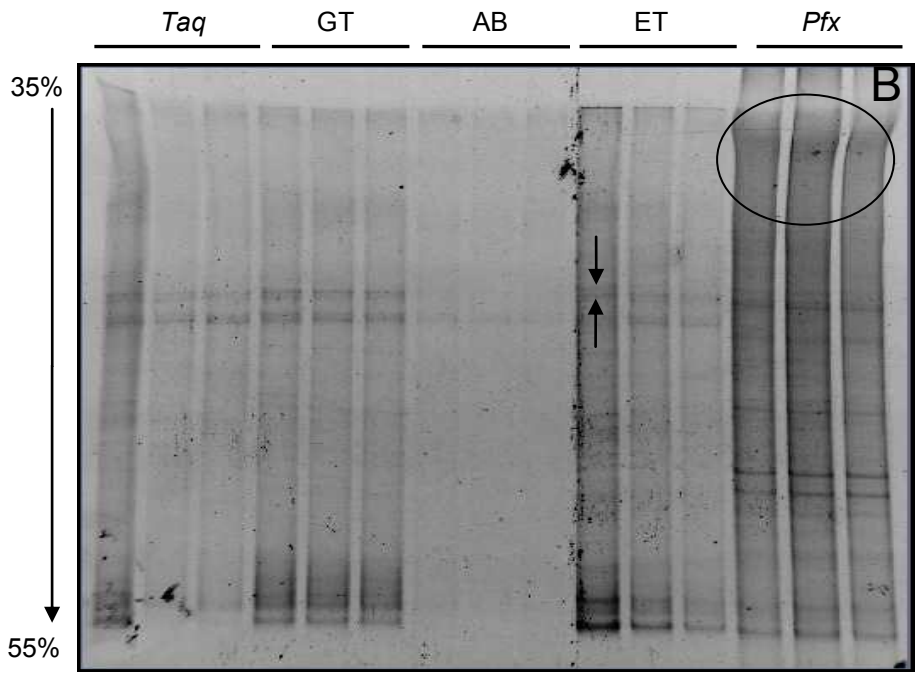
Sample replicate number	Band range per lane	Mean number of bands per lane	*H'* range across sample	Mean *H'* across sample
SS1R	4-19	11.6	1.23-2.88	2.20
SS2R	4-13	9	1.10-2.52	2.03
SS3R	2-25	13.5	0.66-3.16	2.31
SS4R	1-21	10.9	0.61-3.03	1.95
SS5R	1-21	12.1	2.01-2.95	2.03

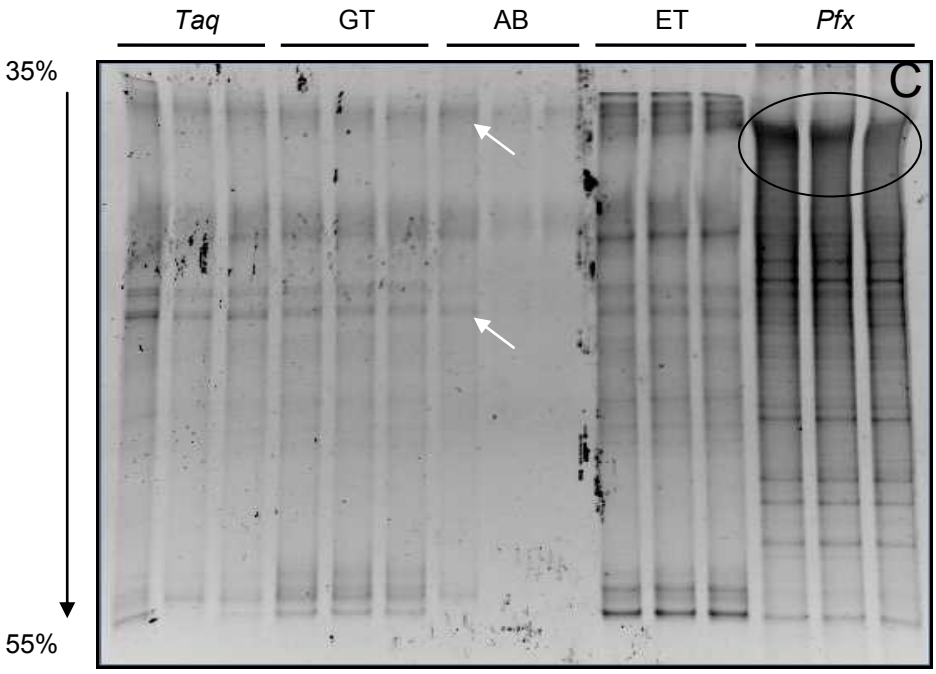
Qualitatively, each DGGE profile generated (i.e., SS1R to SS5R) yielded similar banding patterns from bacterial taxa detected by the five individual DNA pols assessed, including the technical replicates (Fig. 3.2A-E). Applied Biosytems™ AmpliTaq® performance was very poor in comparison to the other DNA pol assessed due to the distinct lack of bands throughout the denaturing gradient, in particular, the bands that were detected are faint (white arrows, Fig. 3.2C). New England BioLabs® Inc. *Taq* and Promega's GoTaq® HS both produced approximate equivalent banding patterns from their technical replicates in all DGGE profiles (Fig. 3.2A-E). Both high-fidelity DNA pol enzymes *Ex Taq*™ HS and Platinum® *Pfx* detected the most abundant bacterial taxa from the dominant members of the community as seen in all gels below resulting in a greater mean band range across all five sample types. Amplification of the V3 rDNA regions performed with *Pfx* did result in more bacterial taxa being detected in SS2R, SS3R, and SS5R. Interestingly, bands were notably absent in the upper 25 % of the denaturing gradient (i.e., near the top of the gel in Fig. 3.2A-E, circled) in almost all *Pfx* technical replicates and thus consequently not detected. TAKARAS BIO INC. *Ex Taq*™ HS in SS1R-SS5R in this regard produced a more widespread detection of bacterial taxa throughout the 35-55 % denaturing gradient utilised (and indeed more numerous specifically in SS1R and SS4R), in addition to taxa that migrated towards the top of the gradient. A global factor in all of the DNA pol enzymes assessed is that band detection in the lower part of the denaturing gradient seemed to be more abundant in the derived *Thermus aquaticus* DNA pol types despite the band number, whereas in *Pfx*, derived from *Pyrococcus* spp. strain KOD1, detected a greater number of bands in the middle

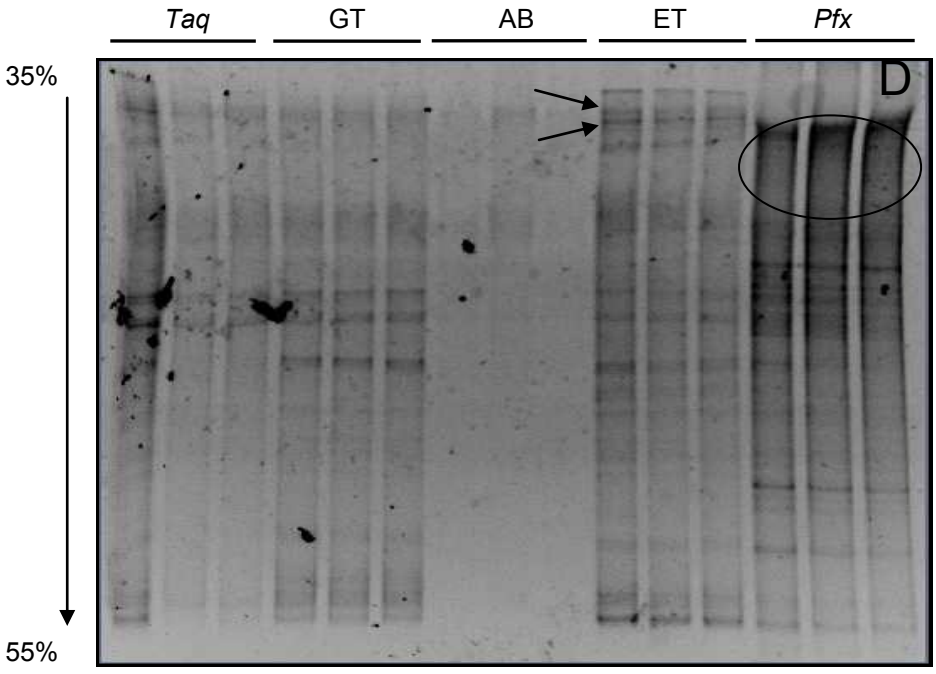
of the gradient. All DGGE profiles generated showed heteroduplex artefacts (i.e., double band formations that migrate through the denaturing gradient very close together) and these have been putatively identified with examples shown in Fig. 3.2A, B, and D. These double bands were common in all *Taq*, GoTaq® HS, and *Ex Taq*™ HS technical replicates across all five sample types.

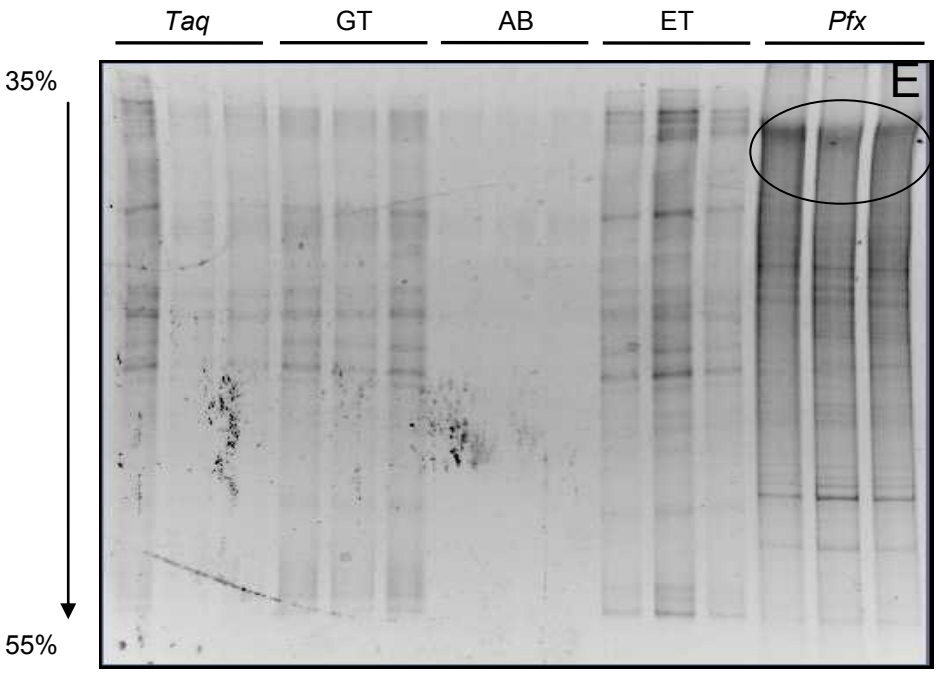
Fig. 3.2A-E: DGGE profiles (35-55 % denaturing gradient) of SS1R-SS5R showing V3 rDNA fragments from all DNA pol technical replicates assessed. SS1R = A; SS2R = B; SS3R = C; SS4R = D, and SS5R = E. Top of the denaturing gradient is shown (35 %) along with the bottom (55 %). Migration direction of the amplicons and denaturing gradient is indicated on the left in gels A-E (arrow). Technical replicates of each DNA pol enzyme are indicated above each DGGE profile (A-E) respectively. Non-detection of bands by *Pfx* in all technical replicates across SS1R-SS5R is circled. Examples of putative artefacts in DGGE profiles are shown (black arrows) whereas detection of taxa by AmpliTaq® is indicated because of faint bands produced in gel profiles (white arrows). AB, AmpliTaq®; ET, *Ex Taq*™ HS; GT, GoTaq® HS.






Taq
GT
AB
ET
Pfx
35%
55%
C


Taq
GT
AB
ET
Pfx
35%
55%
D


Taq
GT
AB
ET
Pfx
35%
55%
E

3.3.2 Statistical analysis from DGGE profiles generated

All DGGE profiles generated and the number of taxa detected by each DNA pol enzyme was then subjected to principal components analysis (PCA) to see if there were any differences between enzyme and sandy soil sample. The first two components were displayed in the outputs (*x*- and *y*-axis) accounting for the cumulative percentage variance explained with SS5R showing the highest percentage variation at 84 %, followed by 80 % (SS4R), 61 % (SS3R), 44 % (SS2R), and finally 59 % (SS1R). All *P*-values derived from the first principal component output by one-way ANOVA (analysis of variation) were significant indicating that there was differences in the number of bacterial taxa detected between the disparate DNA pol enzymes assessed in each DGGE profile (SS1R, $P = < 0.001$, SS2R, $P = 0.001$, SS4R, $P = < 0.001$, and SS5R, $P = < 0.001$) with the exception of SS3R ($P = 0.118$). Furthermore, from the PCA outputs a difference can be clearly seen in the clustering between the disparate types of thermostable polymerases assessed and their technical replicates. Both high-fidelity pols *Ex Taq*™ and *Pfx* exclusively cluster together in all of the DGGE profiles (Fig. 3.3A-E). This strongly suggests that though they detected the most number of bacterial taxa in comparison to the other enzymes, there was still a difference between these two enzyme's performances in some of the soil samples analysed due to spatial distribution. All second principal components analysed were not significant indicating that there was no inter-sample variance between the DGGE profiles SS1R-SS5R examined.

We next wanted to investigate whether there was a difference in bacterial taxa detected by the technical replicates analysed in SS1R-SS5R. Shannon diversity index scores generated by each thermostable DNA pol enzyme technical replicate were calculated and then a one-way ANOVA was performed using Tukey's test to determine which thermostable enzymes were significantly different from each other in terms of their microbial diversity detected. All *P*-values were significant (SS1R-SS5R, $P = < 0.001$) between the H' scores obtained by each DNA pol in each soil sample showing differences in the microbial diversity detected using the five different thermostable enzymes (tables 3.4-3.8). In all five samples analysed, AmpliTaq® thermostable DNA pol was significantly different from the other DNA polymerases assessed (tables 3.4-3.8 ($P = < 0.001$)). However in SS1R, (GoTaq® HS and *Taq*; table 3.4 ($P = < 0.001$)), SS2R (*Taq*; table 3.5 ($P = < 0.001$)) and SS4R (*Ex Taq*™ HS; table 3.7 ($P = < 0.001$)) DNA pol enzymes indicated in brackets were also significantly different to the other in addition to AmpliTaq® based on their H' scores. In SS5R all DNA

pol were significantly different from each other in the bacterial taxa detected (table 3.8 $P = < 0.001$).

Table 3.4: Comparison of *H′* scores generated by each DNA pol replicate and significant differences in diversities detected in SS1R.

DNA pol replicate	***H′* score**	**Tukey's test grouping from ANOVA‡**
Taq	1.86	A
Taq	1.93	
Taq	2.02	
GoTaq® HS	2.15	B
GoTaq® HS	2.42	
GoTaq® HS	2.30	
AmpliTaq®	1.31	C
AmpliTaq®	1.33	
AmpliTaq®	1.23	
Ex Taq™ HS	2.81	D
Ex Taq™ HS	2.79	
Ex Taq™ HS	2.89	
Pfx	2.65	D
Pfx	2.59	
Pfx	2.72	

‡ Tukey's test: DNA pols that do not share a letter are significantly different from each other ($P = < 0.001$)

Table 3.5: Comparison of *H′* scores generated by each DNA pol replicate and significant differences in diversities detected in SS2R.

DNA pol replicate	*H′* score	Tukey's test grouping from ANOVA
Taq	2.17	A
Taq	1.78	
Taq	1.56	
GoTaq® HS	2.33	B
GoTaq® HS	2.34	
GoTaq® HS	2.24	
AmpliTaq®	1.34	C
AmpliTaq®	1.38	
AmpliTaq®	1.10	
Ex Taq™ HS	2.44	B
Ex Taq™ HS	2.35	
Ex Taq™ HS	2.10	
Pfx	2.39	B
Pfx	2.52	
Pfx	2.34	

Table 3.6: Comparison of *H′* scores generated by each DNA pol replicate and significant differences in diversities detected in SS3R.

DNA pol replicate	*H′* score	Tukey's test grouping from ANOVA
Taq	2.34	A
Taq	2.13	
Taq	1.99	
GoTaq® HS	2.38	A
GoTaq® HS	2.39	
GoTaq® HS	2.33	
AmpliTaq®	1.83	B
AmpliTaq®	0.69	
AmpliTaq®	0.66	
Ex Taq™ HS	2.83	A
Ex Taq™ HS	2.79	
Ex Taq™ HS	2.79	
Pfx	3.16	A
Pfx	3.16	
Pfx	3.15	

Table 3.7: Comparison of *H′* scores generated by each DNA pol replicate and significant differences in diversities detected in SS4R.

DNA pol replicate	*H′* score	Tukey's test grouping from ANOVA
Taq	2.25	A
Taq	1.77	
Taq	1.79	
GoTaq® HS	2.28	A
GoTaq® HS	2.16	
GoTaq® HS	2.17	
AmpliTaq®	0	B
AmpliTaq®	0.60	
AmpliTaq®	0	
Ex Taq™ HS	3.0	C
Ex Taq™ HS	3.0	
Ex Taq™ HS	3.0	
Pfx	2.43	A
Pfx	2.24	
Pfx	2.51	

Table 3.8: Comparison of *H′* scores generated by each DNA pol replicate and significant differences in diversities detected in SS5R.

DNA pol replicate	*H′* score	Tukey's test grouping from ANOVA
Taq	2.60	A
Taq	2.29	
Taq	2.18	
GoTaq® HS	2.21	B
GoTaq® HS	2.01	
GoTaq® HS	2.25	
AmpliTaq®	0	C
AmpliTaq®	0	
AmpliTaq®	0	
Ex Taq™ HS	2.78	D
Ex Taq™ HS	2.94	
Ex Taq™ HS	2.54	
Pfx	2.84	E
Pfx	2.84	
Pfx	2.95	

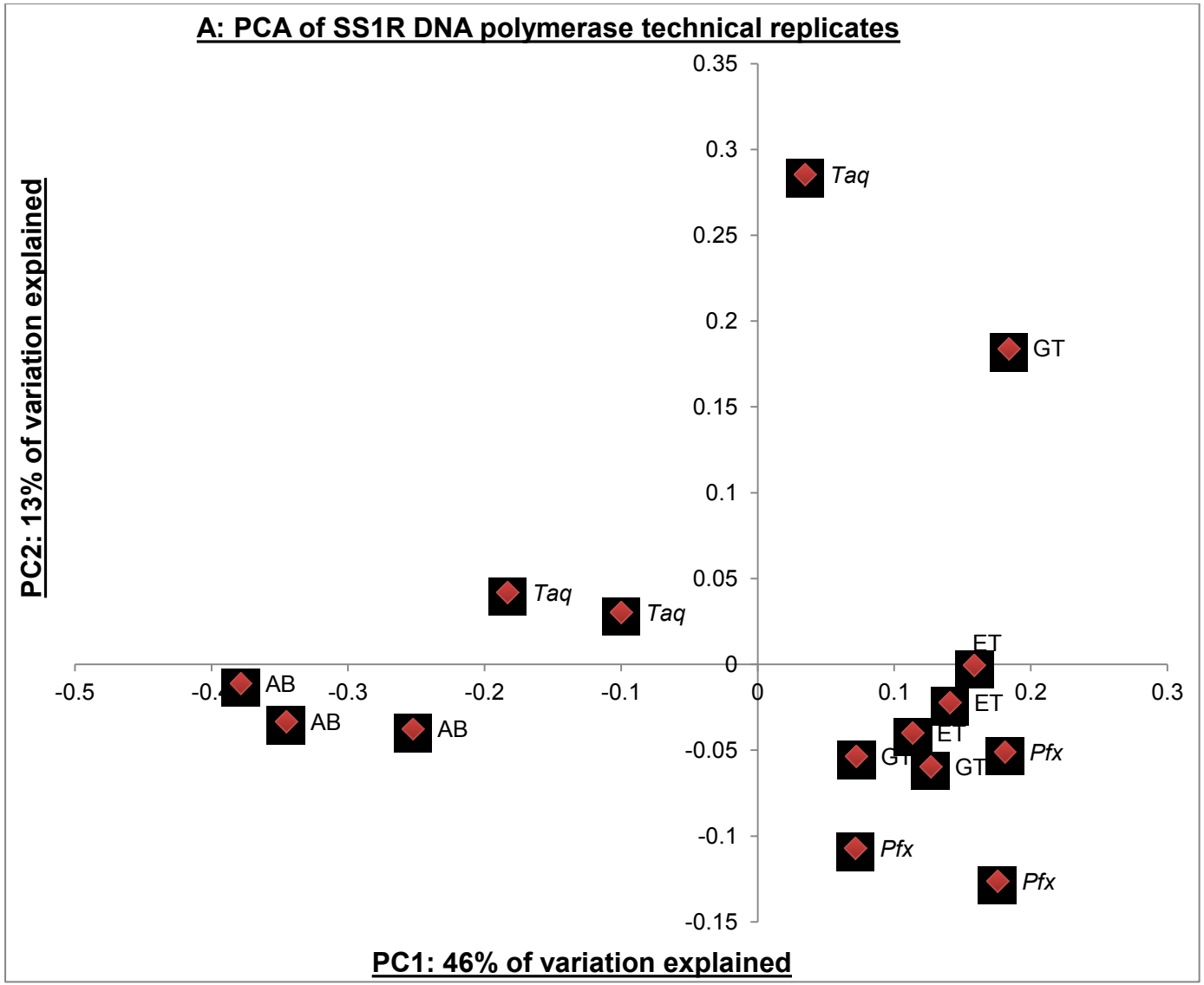
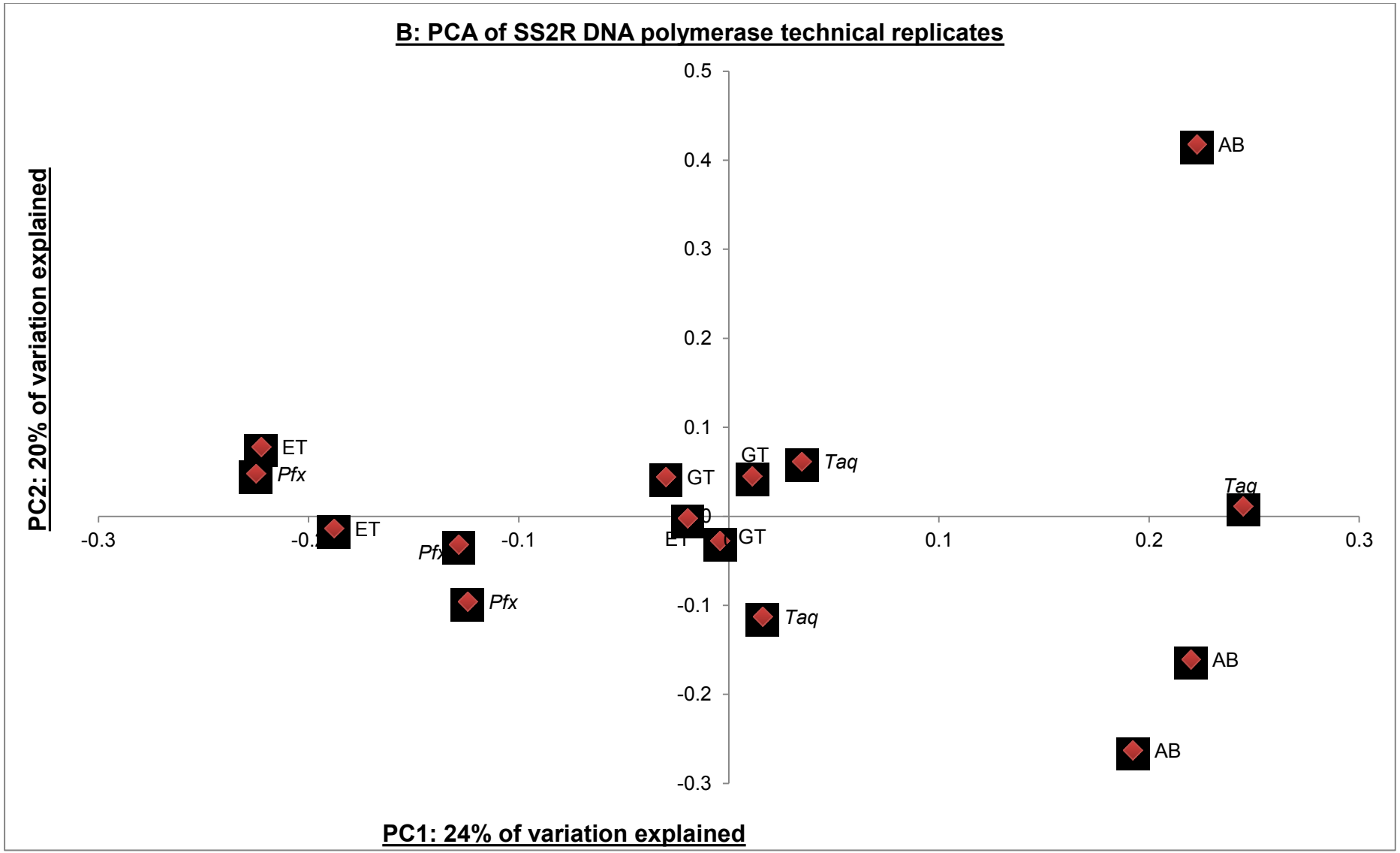
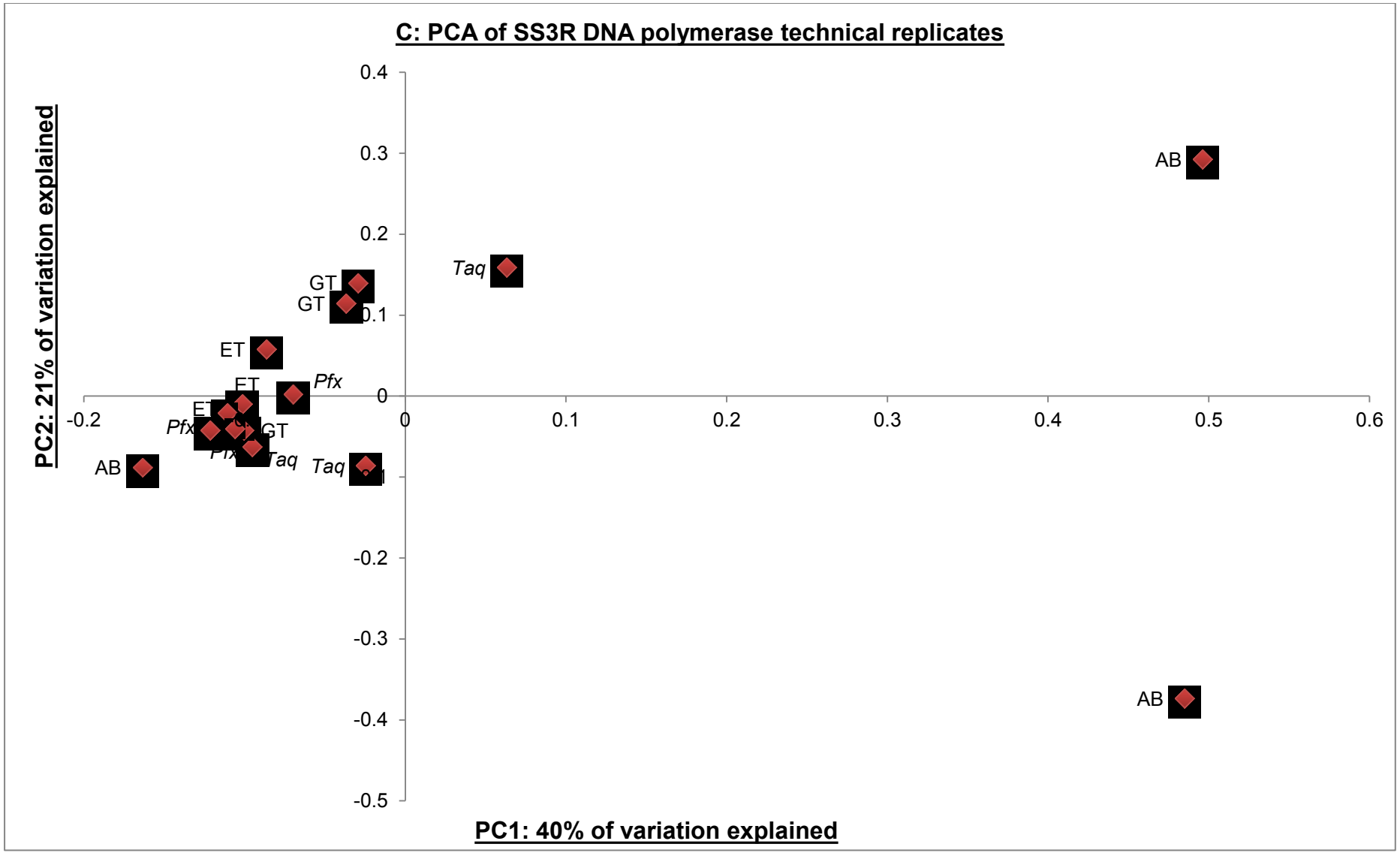
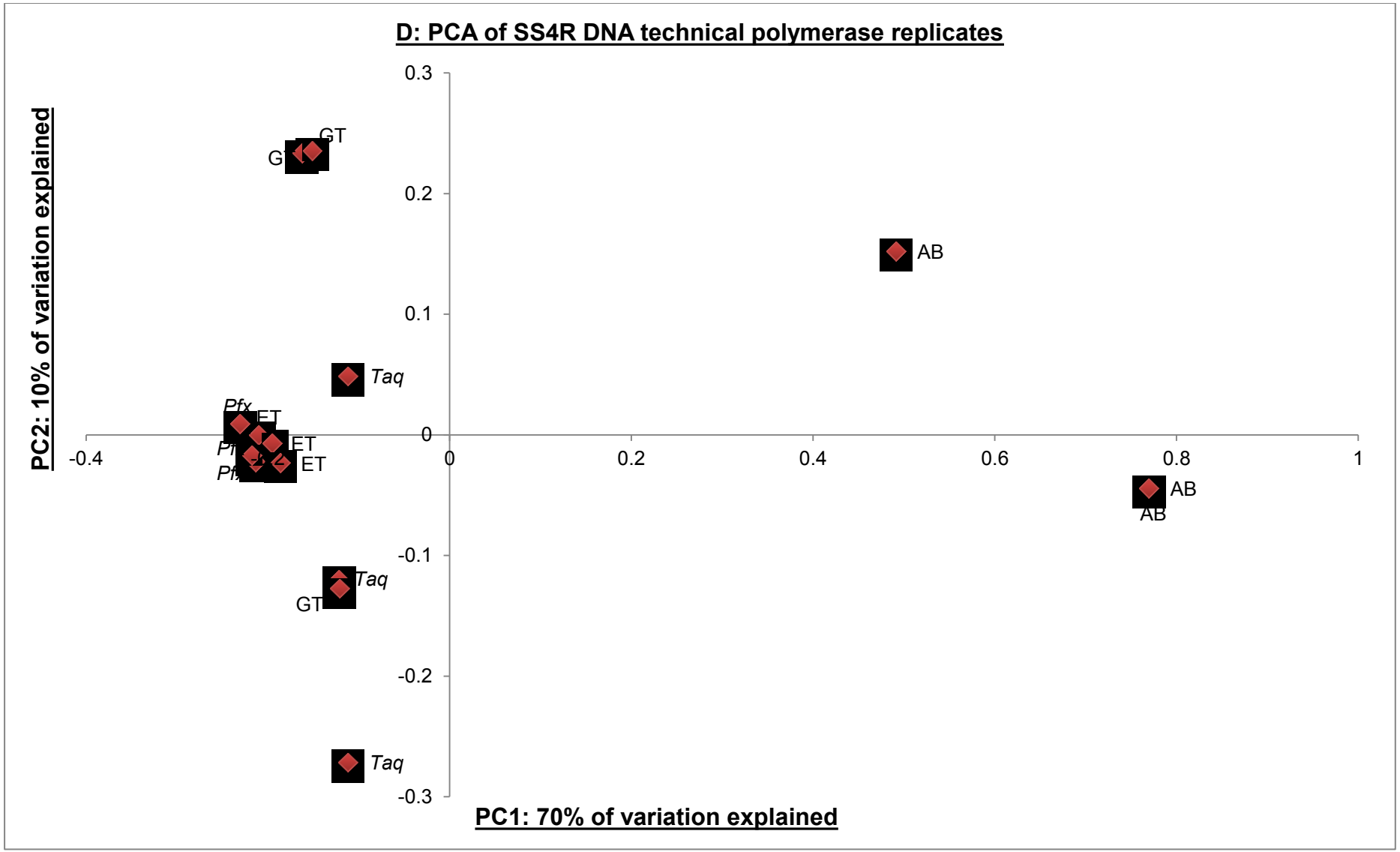
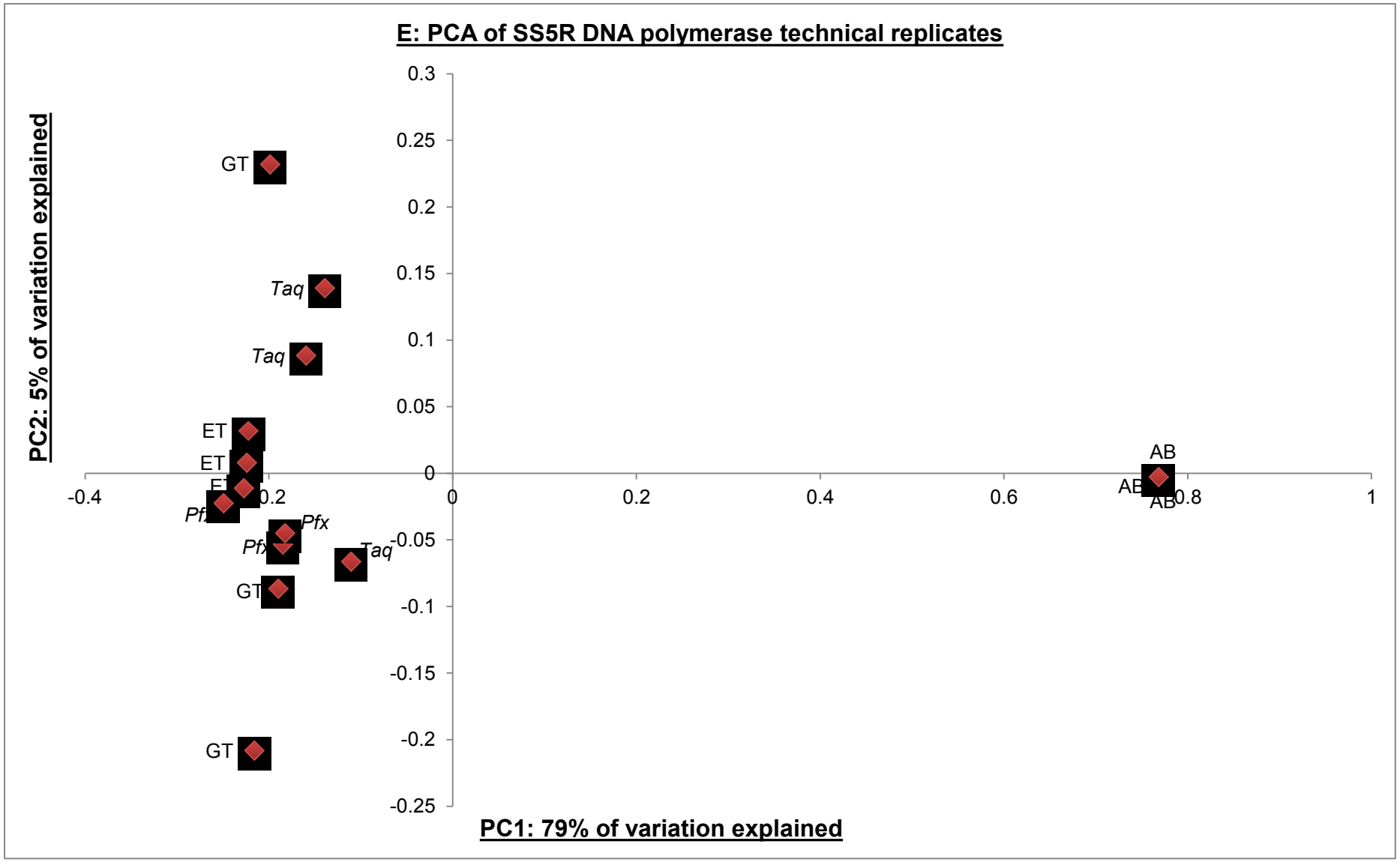


Fig. 3.3A-E: PCA of SS1R-SS5R from each of the DGGE profiles generated and DNA pol technical replicates assessed. SS1R = A; SS2R = B; SS3R = C; SS4R = D; SS5R = E. Each DNA pol technical replicate is indicated in the PCA ouput (AB, AmpliTaq®; ET, *Ex Taq*™ HS; GT, GoTaq® HS; *Pfx*, Platinum® *Pfx*; *Taq*, New England BioLabs® Inc. *Taq*). The *x*-axis (PC1) explains the most variation followed by the *y*-axis (PC2) explaining the second most variation in the PCA output. This variation is shown as two percentages indicated on both the *x*- and *y*-axes respectively.


B: PCA of SS2R DNA polymerase technical replicates
PC2: 20% of variation explained
PC1: 24% of variation explained
0.5
0.4
0.3
0.2
0.1
-0.1
-0.2
-0.3
-0.3
-0.1
0.1
0.2
0.3
AB
AB
AB
ET
ET
ET
GT
GT
GT
Pfx
Pfx
Pfx
Taq
Taq
Taq


C: PCA of SS3R DNA polymerase technical replicates
PC2: 21% of variation explained
PC1: 40% of variation explained
0.4
0.3
0.2
0.1
0
-0.2
-0.3
-0.4
-0.5
-0.2
0
0.1
0.2
0.3
0.4
0.5
0.6
AB
Taq
GT
GT
ET
ET
Pfx
ET
Pfx
GT
Taq
Taq
AB
AB


D: PCA of SS4R DNA technical polymerase replicates
PC2: 10% of variation explained
PC1: 70% of variation explained
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
0
0.2
0.4
0.6
0.8
1
GT
GT
AB
Taq
Pfx
ET
ET
ET
AB
AB
Taq
GT
Taq


E: PCA of SS5R DNA polymerase technical replicates
PC2: 5% of variation explained
PC1: 79% of variation explained
0.3
0.25
0.2
0.15
0.1
0.05
0
-0.05
-0.1
-0.15
-0.2
-0.25
-0.4
-0.2
0
0.2
0.4
0.6
0.8
1
GT
Taq
Taq
ET
ET
AB
AB
AB
Pfx
Pfx
Taq
GT
GT

3.4 Discussion

In this study we have demonstrated that five available commercial thermostable DNA pol enzymes amplifying the V3 region within the bacterial 16*S* rRNA gene can produce DGGE profiles across five sandy soil samples excavated from Longsands Beach, Tynemouth, North East England. However, within the DGGE profiles generated and between the technical replicates of the five DNA pol enzymes assessed significant dissimilarity was exhibited in the detection of the dominant members of the bacterial community in four out of the five sample types. Further, high fidelity DNA polymerases (*Ex Taq* HS™ and *Pfx*) enabled detection of abundant bacterial taxa that eluded detection by the low-fidelity DNA pols, i.e., AmpliTaq®, GoTaq® HS and *Taq*. In all five samples analysed by DGGE, AmpliTaq™ DNA pol performance was significantly different ($P = < 0.001$) in detection of bacterial taxa in sandy soil (tables 3.4-3.8).

From the results presented above, a discussion of the data and each respective DNA pol performance must be approached with caution in respect to other PCR-DGGE mediated studies and the inherent bacterial community because of several underlying potential biasing factors: (i), PCR amplification can be inhibited by co-extracted contaminants from the sample type (i.e., humic substances in soil) (ii), differential amplification — does the amplification of 16*S* rDNA produce a pool of either homologous or heterogeneous template molecules? (iii), PCR fragment artefactual formation (iv), contaminating DNA, and (v), rRNA gene region (*rrn* operon) variability within the community amplified (von Wintzingerode et al., 1997). The high-fidelity polymerases, (*Ex Taq*™ HS and Platinum® *Pfx*) gave significantly ($P = < 0.001$) higher bacterial taxa detection than the lower fidelity alternatives used in this study. In particular is the amplification of the V3 region by both *Ex Taq*™ HS and *Pfx* which resulted in a higher proportion of GC-rich bacterial taxa detected (Fig. 3.2A-E) such as the Actinobacteria. Conversely where one expects to find bands with a low GC-ratio typical of the Proteobacteria it was possible to differentiate between *Pfx* and *Ex Taq*™ HS PCR products. The latter appeared much more effective in amplifying low GC-ratio 16*S* rRNA genes as seen in all of the gels produced (Fig. 3.2A-E; circled). In three soils (SS2R, SS3R, SS5R) the diversity of bacteria detected by *Pfx* was greater than that detected by *Ex Taq*™ HS (tables 3.5, 3.6, and 3.8) Whereas in SS1R and SS4R bacterial diversity detected using *Ex Taq*™ HS was higher than that of *Pfx* (tables 3.4 and 3.7). However, in comparing *Ex Taq*™

HS and *Pfx* high-fidelity pols only significant differences between the two were observed in SS4R and SS5R ($P = < 0.001$). In SS1R, SS2R, and SS3R there was no significant difference.

Production of PCR artefacts and chimeras is a confounding factor that may lead to an over-estimation of the microbial diversity present in the sample analysed, however, the formation of these anomalies is not uncommon in DGGE (Muyzer, 1999). Chimera formation is generated by incompletely synthesised extended DNA fragments annealing together producing closely related sequences generating recombinants between starting templates (Judo et al., 1998, Bradley and Hillis, 1997). Differentiation between original haplotypes and chimeras generated can be problematic resulting in occasionally an overestimation of the community diversity in microbial ecology studies (von Wintzingerode et al., 1997, Hugenholtz and Huber, 2003, Berney et al., 2004). A study reported several years ago has tried to circumvent this problem by introducing an additional extension time of 30 min reducing the formation of double bands in PCR-DGGE analysis in environmental microbial diversity PCR (Janse et al., 2004). This longer extension time was implemented here in all DNA pol technical replicates carried out at the end of each PCR cycle. GC-clamp attachment to the forward primer, although designed to improve sequence heterogeneity from the pool of amplicons in analysis by DGGE, can induce effects such as G quartet or four-stranded tetraplex formation caused by multiple adjacent G residues (Poon and Macgregor, 1998, Poon and Macgregor, 2000). Further, a recently published study suggested the procurement of enough oligonucleotides incorporating a GC-clamp in a single batch for a project to avoid the GC-clamp sequence itself actually affecting DGGE profiles due to differences in performance between repeat syntheses of GC-clamp oligonucleotide primers from disparate batches (Rettedal et al., 2010).

Duplex banding patterns seen in the DGGE profiles (Fig. 3.2A, B, and D) could also be attributed to the formation of spurious PCR products after the addition of the DNA pol to the reagent master mix (MM). This can be circumvented by the induction of either a manual or automatic HS; with the exception of *Ex Taq*™ HS, GoTaq® HS and Platinum® *Pfx*, (all bound to a proprietary antibody) both AmpliTaq® and *Taq* were introduced into the master-mix via a manual HS procedure, but despite this process, poor AmpliTaq® performance was unequivocal in bacterial taxa detection unlike that of its counterpart *Taq*. A previous study assessed the impact of DNA polymerase bound to a proprietary antibody on the resulting

PCR products synthesised. This demonstrated an increased specificity, sensitivity, and yield, in addition to reducing the formation of spurious PCR products before the PCR was actually initialised (Sharkey et al., 1994).

Assessment of the microbial diversity in sandy soil samples using the *T. aquaticus* derived DNA pol enzymes (i.e., *Taq*, GoTaq® HS, and AmpliTaq®) produced similar banding patterns in most replicates across SS1R-SS5R. However, AmpliTaq® amplification of pooled V3 rDNA amplicons produced very faint bands in the denaturing gradient (Fig. 3.2C; white arrows). It is questionable if this is a true reflection of AmpliTaq®'s capability in comparison to the other DNA pols. Further optimisation of AmpliTaq® is probably required to draw satisfactory results and conclusions. Detection of hypothetical GC-rich bacterial taxa was noticeable in both GoTaq® HS and *Taq*, although less so than in *Ex Taq*™ HS and *Pfx*.

Another mitigating factor in the variances of DGGE profiles generated from the commercially available thermostable enzymes assessed here, concerns the DNA pol property of fidelity. The DNA pols *Taq*, GoTaq®, and AmpliTaq® carry out DNA replication and repair with a low degree of fidelity and have a low proofreading capacity in comparison to *Ex Taq*™ and *Pfx*. In relation to this, it can be suggested that the formation of PCR-mediated recombination or chimeras, giving rise to heteroduplex PCR products, would increase when using *Taq*, GoTaq® HS, or AmpliTaq® when assessing the microbial diversity in any given environmental type in PCR-DGGE studies. It is worth noting that despite the higher mean band ranges and H' scores produced by both *Ex Taq*™ HS and *Pfx*, the risk of artefactual PCR products is decreased compared to their lower-fidelity counterparts. Surprisingly, it has been previously shown that even high-fidelity DNA pols insert incorrect nucleotides with similar efficiencies to that of naturally occurring low-fidelity polymerases (Beard and Wilson, 2003). This finding concludes that right from selection of DNA pol in PCR-DGGE mediated investigations, there will always be an inherent bias, and as thus, this must be taken into account from the data presented.

3.5 Conclusions

In summary, all DNA pol enzymes employed in this study could have been optimised further, in particular AmpliTaq®, raising concerns over its true performance in the microbial community detection from the sample types collected herein. What is apparent, and significant in four out of the five samples analysed ($P = < 0.001$), is that from the DNA pol technical replicates assessed is that PCA shows that the higher-fidelity pols *Ex Taq*™ HS and Platinum® *Pfx* cluster together explaining some of the variance observed. This variance can be attributed between the other DNA pols assessed, in addition to greater bacterial taxa detection and H' scores generated. From the data presented, comprising both the multivariate analysis and DGGE profiles generated, *Ex Taq*™ HS DNA pol proved the most effective in best representing the microbial communities present in the samples analysed by DGGE despite the inherent limitations of this approach outlined above. Thus *Ex Taq*™ HS will be implemented in the PCR-DGGE mediated studies in two chronic respiratory tract disorders presented further in this body of work.

Chapter Four: PCR-DGGE study of a cross-sectional cohort with non-cystic fibrosis bronchiectasis

4.1 Abstract

The aim was to investigate the polymicrobial communities in sputum samples derived from an adult non-cystic fibrosis bronchiectasis cohort that consisted of 70 individuals (25 males and 45 females). Twenty patients had symptoms consistent with an exacerbation, the remainder were clinically stable. DNA was extracted from sputum samples of all patients. Universal primers were used to amplify fragments both within the 16*S* and 28*S* rRNA genes. Amplicons were analysed by denaturing gradient gel electrophoresis. Demographic and culture data were used in constrained ordination analyses to identify any significant associations between these data and changes in the sputum microbiota. Standard culture indicated that 19 (27 %) sputum samples were negative for recognised pathogens. Molecular analyses demonstrated these samples contained a bacterial diversity (mean = 7 taxa) not significantly different to culture positive sputum. There was a significant difference between the bacterial community structure in the sputum samples of exacerbated patients compared to those that were clinically stable (P = 0.002). Antibiotic administration within 1 month of sampling caused no significant changes to the microbial community investigated. *Haemophilus influenzae* carriage is identified as a significant driver (P = 0.006) of change in the microbial community structure. Further, *H. influenza*e was not identified in samples (n = 12) that harboured *Pseudomonas aeruginosa.* Consistent with earlier studies, the persistent presence of *P. aeruginosa* (n = 23) determined by culture was significantly correlated with reduced lung function (P = < 0.001). In adult non-CF bronchiectasis patients bacterial lung communities show significant differences between exacerbation and clinically stable states and also when *H. influenzae* is present. Antibiotic administration does not significantly alter these microbial communities. Persistent colonisation by *P. aeruginosa* is significantly associated with reduced lung function, and is negatively correlated with *H. influenza*e carriage.

4.2 Background

Bronchiectasis is a highly debilitating disease causing severe pulmonary infections and lung function decline resulting in chronic morbidity, and in some cases, premature mortality (King et al., 2005). The disease itself was initially characterised in 1819 by René Théophile Hyacinthe Laënnec in which from his writings, he described that "This affection of the bronchioles is always produced by chronic catarrh, or by some other disease attended by long, violent, and often repeated fits of coughing" (Laënnec, 1962). Presently, bronchiectasis is defined by irreversible abnormal dilatation of one or more bronchi, with chronic airway inflammation, associated with chronic cough and sputum production, recurrent chest infections, and airflow obstruction (Barker, 2002, Cohen and Sahn, 1999, Wilson and Boyton, 2006). In terms of a primary phenotype, bronchiectasis exists in two forms; (i) cystic fibrosis (a genetically inherited disease resulting in a severe form of bronchiectasis (Strausbaugh and Davis, 2007)), and (ii), non-cystic fibrosis bronchiectasis (nCFBR). Contributing factors that induce nCFBR can be physical obstruction or post infectious damage, genetic defects (as observed in cystic fibrosis (CF)), abnormal host defence mechanisms or autoimmune disease, in many cases nCFBR is idiopathic (Pasteur et al., 2001).

Diagnosis of nCFBR using high-resolution computed tomography (HRCT) reveals varying structural and anatomic phenotypes within either one lobe or both lobes of the lung, pending local or more commonly general distribution of the disease in the lower lobes (King et al., 2006). These characteristic phenotypes defined by Reid described three main subtypes of bronchiectasis; (i), smooth dilation of the bronchi giving rise to the tubular form (ii), dilated bronchi populated with multiple indentations — varicose phenotype and (iii), cystic, dilated bronchi terminating in blind sacs (Reid, 1950). As opposed to HRCT, pathology of bronchiectasis lung tissue specimens by Whitwell resulted in the addition of three sub-types of the bronchiectasis phenotype: (i), follicular (ii), saccular and (iii), atelectatic. From his findings, infiltration of the bronchial wall with inflammatory cells in conjunction with the loss of elastin, and in more severe cases, muscle and cartilage injury was associated with bronchial dilatation (Whitwell, 1952). In conjunction with HRCT and Whitwell's description, the dominant form of bronchiectasis disease today is the follicular sub-type corresponding to the tubular phenotype. In this form, as Whitwell had previously shown earlier; in the small airways lymphoid follicle formation by the infiltration of inflammatory cells in the bronchial

walls is extensive, causing obstruction of the small airways. Conversely, in the larger airways, dilation is followed by the loss of elastin, cartilage and muscle (King, 2011b). Paradoxically, studies have shown that in bronchiectasis individuals, lung function decline occurs gradually with loss of forced expiratory volume in one second (FEV_1), despite the characteristic dilatation of the airways (King et al., 2005, Martínez-García et al., 2007, Twiss et al., 2006). Explanation of this paradox is demonstrated by Whitwell's original pathology findings demonstrating that during large airway dilation, the small airways were obstructed; net effect of the pulmonary tree is airflow obstruction as composition of this is in the small airways (King, 2011a). Pulmonary airflow obstruction in bronchiectasis caused by structural anatomic changes is critical as they adversely affect normal airway clearance and impede innate immunity mechanisms (Morrisey, 2007). Indeed, bronchiectasis pathophysiology defines the impairment, control, prevention, and resolution of respiratory tract diseases. Cole's original paradigm in bronchiectasis disease states that a vicious circle of recurrent or persistent airway infection and inflammation promotes additional lung tissue structural insults predisposing bronchiectasis individuals to prolonged infection — a self-promoting cycle (Cole, 1986).

Chronic airway infection is strongly associated with bronchiectasis disease and this appears to contribute to the underlying pathogenesis of the disease, with progressive lung damage resulting from recurrent bacterial infections and inflammatory responses (Wilson and Boyton, 2006). Previous studies investigating the bacteriology of nCFBR from several types of clinical samples have identified common lower respiratory tract pathogens including *Haemophilus influenzae*, *Pseudomonas aeruginosa*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*; other less frequently encountered isolates are *Staphylococcus aureus* and non-tuberculosis mycobacterial (NTM) species (Angrill et al., 2002, King et al., 2007, Pasteur et al., 2001, Nicotra et al., 1995). *S. aureus* involvement in nCFBR is uncommon and repeated culture isolation from sputa is suggestive of cystic fibrosis, particularly in younger patients (King, 2011a). When using sputa to investigate the aetiology of nCFBR infection, both *H. influenzae* and *P. aeruginosa* are the most commonly cultured pathogens with many isolated strains showing significant antibiotic resistance (King, 2011a, Angrill et al., 2002). Infection with *H. influenzae* in the lower airways in nCFBR is almost always associated with the non-typeable strain of this species. A normal resident of the upper respiratory tract (URT) in most persons, non-typeable strains lack a polysaccharide capsule where recurrent or

chronic lower airway infection with non-typeable *H. influenzae* (NTHI) in nCFBR is common place (Grimwood, 2011). Persistence of NTHI in the lower airways is due to several mechanisms such as antigenic drift, protease secretion, expression of multiple adhesion molecules, and intracellular survival in either host lung macrophages or epithelial cells (Murphy et al., 2009). Indeed, infection with NTHI is a dynamic process as different strains of this organism can infect bronchiectasis patients sequentially as previously shown in CF (Román et al., 2004). Furthermore the lung bronchi have been shown to exhibit a dynamic turnover of lower respiratory tract (LRT) pathogens; the acquisition of new bacterial strains in bronchiectasis has been investigated in which patients were continually colonised with *Moraxella catarrhalis*, but strains of this bacterium were shown to have a continuous turnover every 2-3 months (Klingman et al., 1995).

As opposed to *H. influenzae*, and the other bacterial isolates above, *P. aeruginosa* colonisation of the URT is infrequent in healthy individuals as it is rapidly cleared from the lower airways (Garau and Gomez, 2003). Indeed, *P. aeruginosa* infection with nCFBR in children is rare; however, in older individuals presenting with bronchiectasis, infection with *P. aeruginosa* is much more common. Individuals with nCFBR harbouring no pathogens showed the mildest disease whereas isolation of *P. aeruginosa* had the most severe bronchiectasis (King et al., 2007). Concurrently, isolation of *H. influenzae* tended to be from patients with moderately impaired pulmonary function (King et al., 2007). *P. aeruginosa* is widely dispersed throughout the environment and is a superb opportunistic respiratory pathogen able to acclimatise to the niche micro-environment of the lung as it is particularly adapted to binding to the damaged epithelial lining of the pulmonary tree in chronic respiratory diseases (de Bentzmann et al., 1996), but more specifically in CF. Most of our understanding of *P. aeruginosa* and its mechanisms of infection in individuals presenting with nCFBR is through extensive work carried out in CF where it is the most important pathogenic isolate (de Vrankrijker et al., 2010). Infection with *P. aeruginosa* in CF is comprised of three phases: no infection, intermittent infection, followed eventually by chronic infection, in which the pathogen undergoes gradual mutational changes in its bacterial genome in response to the micro-environmental challenges in the CF lung (Li et al., 2005). One of the major mutational changes in *P. aeruginosa* in the CF lung is the development from a non-mucoid to mucoid phenotype commonly isolated by culture in chronically infected patients (Foweraker, 2009). Establishment of this mucoid phenotype

plays a major role in phagocytic evasion, increased tolerance to toxic oxygen free radicals mediated by neutrophils and resistance to antibiotics (Murray et al., 2007). The production of biofilms by *P. aeruginosa* is a critical factor in the treatment of CF patients, as the bacteria within them are inherently difficult to eradicate as antibiotics bind to the extracellular matrix as opposed to penetrating the inner matrix in addition to exhibiting increased resistance to the host immune system (Foweraker, 2009) (Davies and Bilton, 2009). Biofilm formation subsequently leads to persistent infection thus resulting in severe damage to the lung tissue architecture.

Chronic infection in nCFBR with *P. aeruginosa* is associated with an increase in airflow obstruction, sputum production, hospitalisations, in addition to extensive lung disease and worsening in quality of life in patients' afflicted (King, 2011b, Martínez-García et al., 2007, Wells et al., 1993). As a result of this, the presence and persistence of *P. aeruginosa* has been identified as a marker of bronchiectasis severity, although it remains unclear whether this is linked to accelerated lung function decline (Davis et al., 2006). Frequent exacerbations experienced by bronchiectasis patients may contribute to the progressive decline of lung function (Martínez-García et al., 2007) though the cause of these remains poorly understood. Exacerbations are frequently managed with antibiotics, however, viral infections may also be significant in many cases but their role requires clarification (King, 2011a). In CF bronchiectasis, there is a significant contribution to exacerbations by fungal pathogens (Bouchara et al., 2009). However, their role, if any, in nCFBR remains to be elucidated.

In this study our aim was to undertake a community level investigation of the microbiome associated with the nCFBR lung, to characterise their diversity and structure, and to determine whether differences in the communities reflect the clinical status of the patient, the presence or absence of culturable pathogens or show significant changes in response to the interventions used in the clinic to manage patient outcomes.

4.3 Results

4.3.1 Patient cohort analysis

Seventy patients (25 male, 45 female) were enrolled in this study (App. 3), both consisting of clinically stable and exacerbating outpatients currently diagnosed with nCFBR at time of sampling. The mean FEV_1 derived was 1.47 L (± 0.78 L) with a mean age of 61.6 ± 12.9 years across cohort. Between the males and females comprising cohort, the absolute FEV_1 values were significantly higher in males 1.84 vs. 1.26 litre ($P = 0.002$). However, there was no significant difference in the age ($P = 0.492$) or the FEV_1% predicted between the sexes ($P = 0.785$). Similarly, the distribution of genders between the culture-negative, exacerbated, non-exacerbated, *H. influenzae* and *P. aeruginosa* colonisation states were not significantly different to the overall sex ration of the cohort. For 61 of the 70 patients, data on the number of exacerbations reported in the preceding 12 months was available. Frequent exacerbators were defined as those patients who had greater than 3 episodes over this period, 38 patients in the cohort satisfied this criterion**.** At the time of sample collection 20 patients reported symptoms consistent with an exacerbation defined as per the British Thoracic Society (BTS) 2010 bronchiectasis guidelines by the presence of increased cough, malaise, increased sputum volume and purulence (Pasteur et al., 2010). A summary of the patient demographic data is shown in table 4.1.

Table 4.1: Summary of patient demographic data.

	All patients (*n* = 70)	**Non-exacerbated sub-cohort (*n* = 50)**	**Exacerbated sub-cohort (*n* = 20)**
Demographic data:			
Age (years)	61.6 ± 12.9	61.2 ± 12.9	62.5 ± 13.4
Female population in cohorts (%)	45 (64.3)	30 (60)	15 (75)
FEV_1 (L) in both genders: Male population Female population	1.47 1.84a† 1.26b	1.46 1.86a 1.17b	1.50 1.77a 1.41b
FEV_1% predicted	58.3 %	56.3 %	64.9 %
Frequent exacerbators (%) (*n* = 61§)	38 (54)	26 (52)	12 (60)
Culture-negative (%)	19 (27)	11 (22)	8 (40)
H. influenzae colonisation (%)	12 (17.1)	7 (14)	5 (25)
P. aeruginosa colonisation (%) Never isolated Intermittently isolated Persistent isolation	 28 (40) 17 (24) 25 (36)	 17 (34) 12 (24) 21 (42)	 11 (55) 5 (25) 4 (20)
Recent Abx¿	16 (23)	10 (20)	6 (30)

§ Frequent exacerbation data was only available for 61 patients in total cohort.

¿ Antibiotic treatment within the last month other than colomycin or azithromycin.

† FEV_1 (L) values in male and female populations followed by different letters are significantly different ($P = <0.05$).

4.3.2 Microbial culture analysis

In whole cohort, 51 patients (73%) from the sputa examined were culture-positive for pathogenic microorganisms; the remainder being classified as either no growth or culture identified mouth flora only (additionally defined as culture-negative because of non-isolation of associated respiratory pathogens). The most common pathogenic organisms isolated from sputa were *P. aeruginosa* (PA) found in 39 % and *H. influenzae* (HI) in 17 % of patients respectively, however, there were no instances of both these taxa being found within a single sputum sample. Only three clinical samples were culture positive for fungal species:

Aspergillus fumigatus was isolated from patient 32 and *Candida* spp. both from patients 8 and 44 respectively.

Assessment of lung function showed that patient's culture negative for pathogens, or those harbouring HI, had similar values. But these were significantly higher (P = 0.0001 and P = 0.0002 respectively) than individuals whose sputum was positive for PA isolation. From the medical records examined, 25 individuals were culture-positive for PA isolation from all previous sputum samples submitted for microbiological testing; these patients were defined as persistently colonised. A further 18 individuals were intermittently colonised and 28 were culture-negative for PA isolated from all previously submitted sputum samples. Following these criteria, we next examined the relationship between the presence and persistence of PA infection and forced expiratory volume in one second predicted (FEV_1% predicted). This demonstrated a significant reduction in FEV_1% predicted (P = < 0.001) between those ever and those never colonised by PA sub-groups. Further, FEV_1% predicted lung function was significantly reduced in persistently versus intermittently colonised (P = 0.004) and persistently versus never colonised patients (P = < 0.001). However, FEV_1% predicted was not significantly different between those with intermittent PA carriage and those where PA had not been isolated (P = 0.1). In patients culture-positive for HI the FEV_1% predicted was significantly higher than the FEV_1% predicted of individual's chronically infected with *P. aeruginosa* (P = < 0.001). Patients experiencing frequent exacerbations (i.e., > 3 exacerbations per annum; n = 38) were predominantly culture-positive for the presence of known lung pathogens (71 %). Within this sub-group of exacerbating individuals, 50 % were colonised with PA and 10.5 % with HI but co-isolation of these two respiratory pathogens was never observed. Pulmonary function (FEV_1% predicted) was not significantly different between patients who frequently exacerbated and the remaining individuals that did not.

4.3.3 Ecological analysis of bacterial community

The bacterial community profiles generated from the PCR-DGGE analysis produced 45 distinct band classes with a mean of 8.4 bands per lane (band range 1-18 per lane) (Fig. 4.1A-G). Each individual DGGE band was presumed to be a single bacterial taxon and was used as a measure of species richness. The mean bacterial Shannon diversity (H') was 1.53 (range 0-2.55) and the mean species evenness (J') of the bacterial communities was 0.78 (range 0.197-0.992). Species evenness is a biodiversity measure used to ascertain how equal the bacterial

community is in this instance via quantification; whereas the *H′* is a measure of both species richness and evenness in the community analysed. Analysis of variance on the entire cohort's community profiles showed that there was no significant difference associated with the number of taxa, community diversity or evenness between those sputum samples where there was a self- reported exacerbation. Similarly, there were no significant differences between taxa or community diversity indices in patients who were never, intermittently or persistently colonised with *P. aeruginosa* or where *H. influenzae* could be isolated from sputa samples.

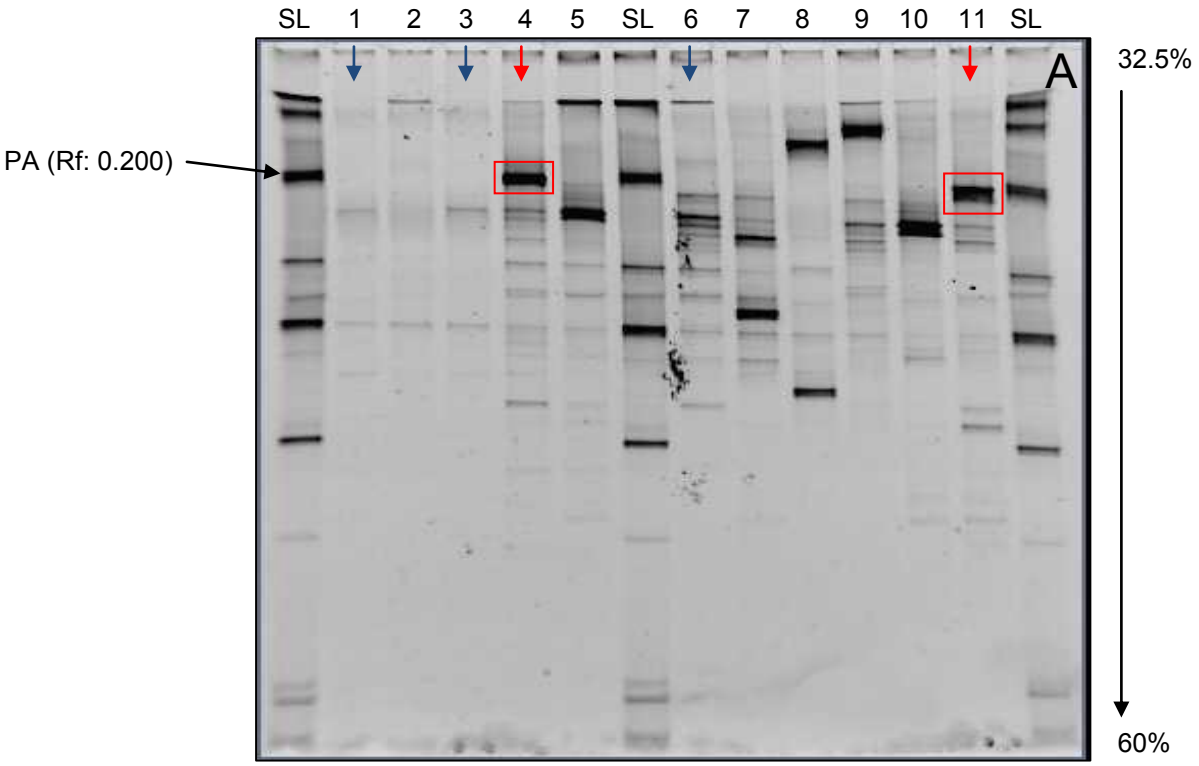
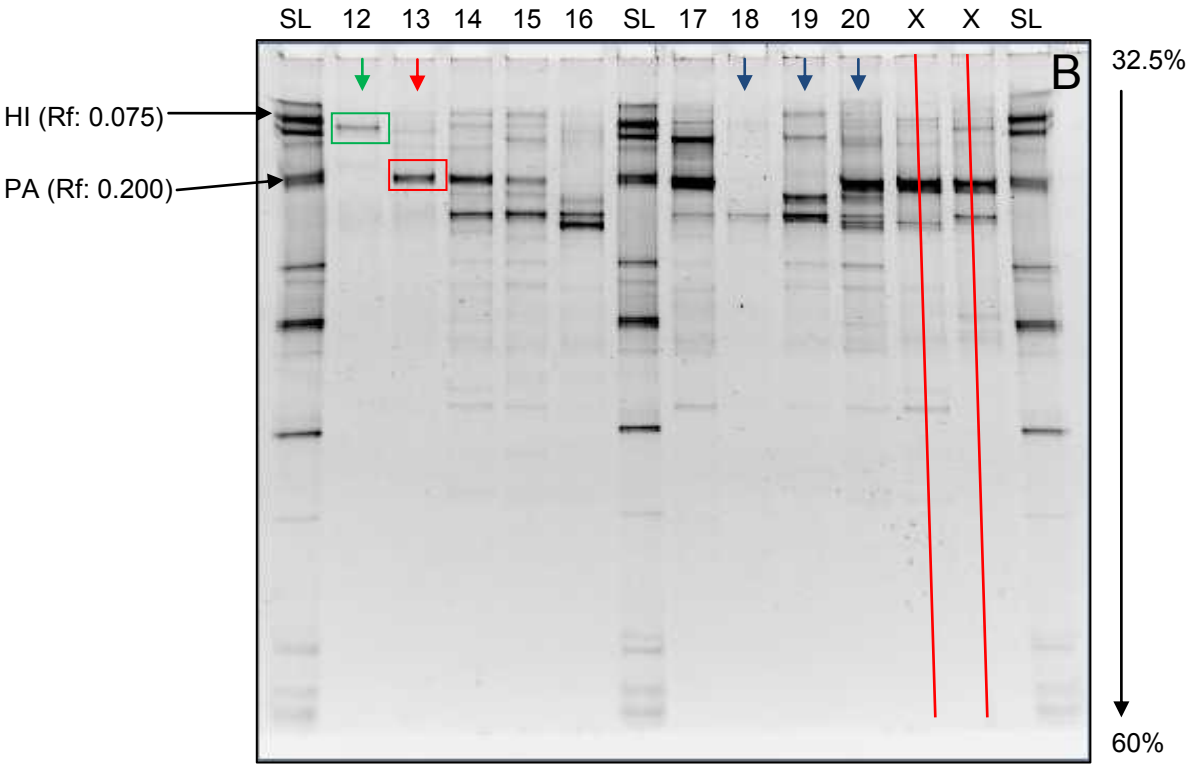
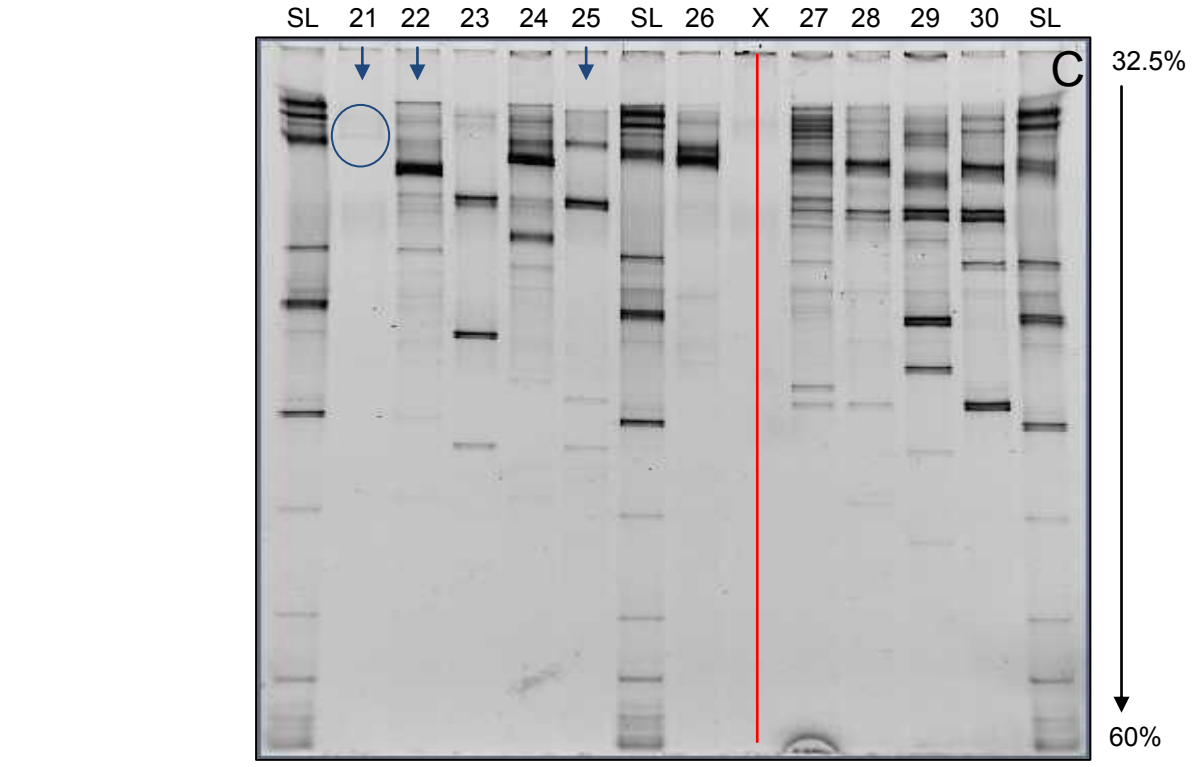
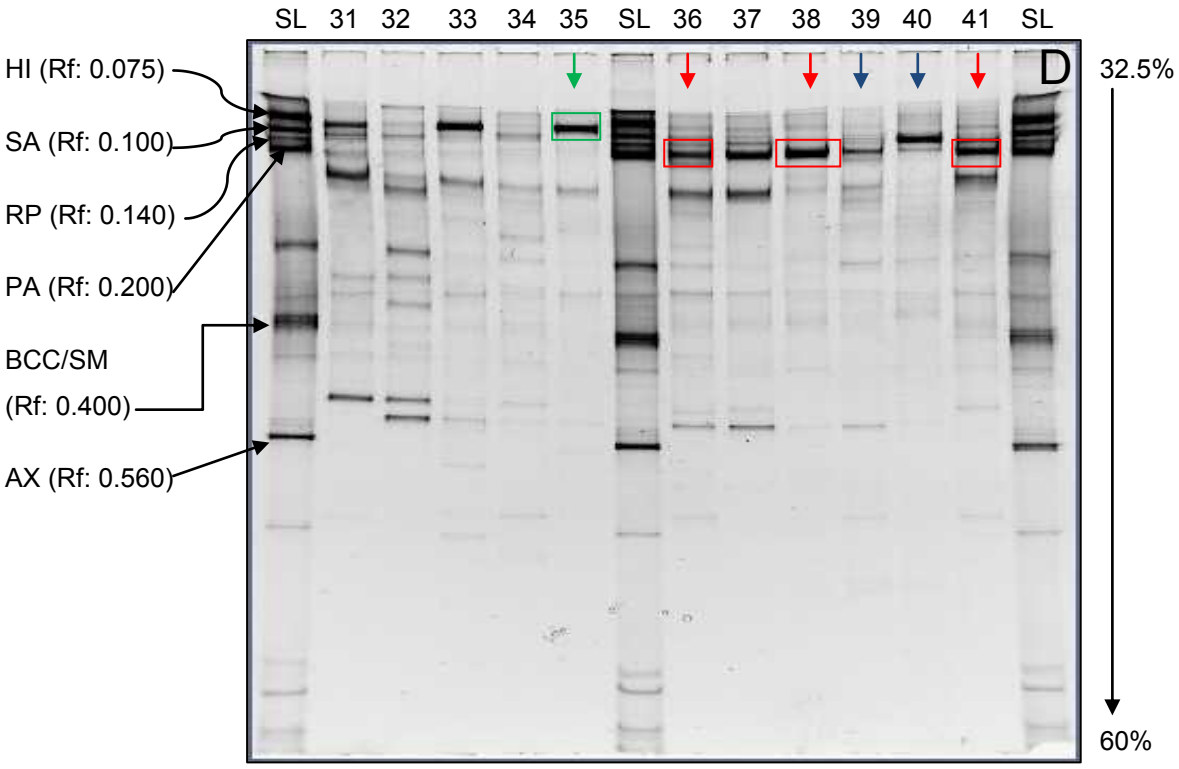
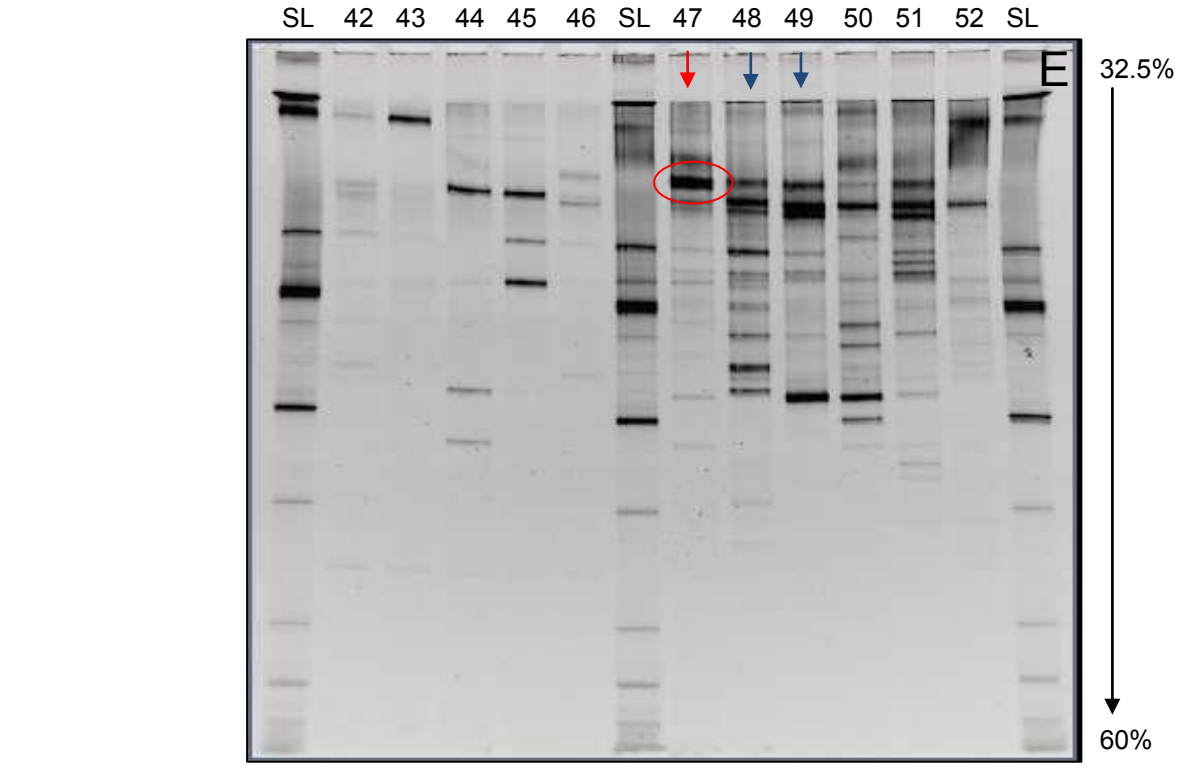


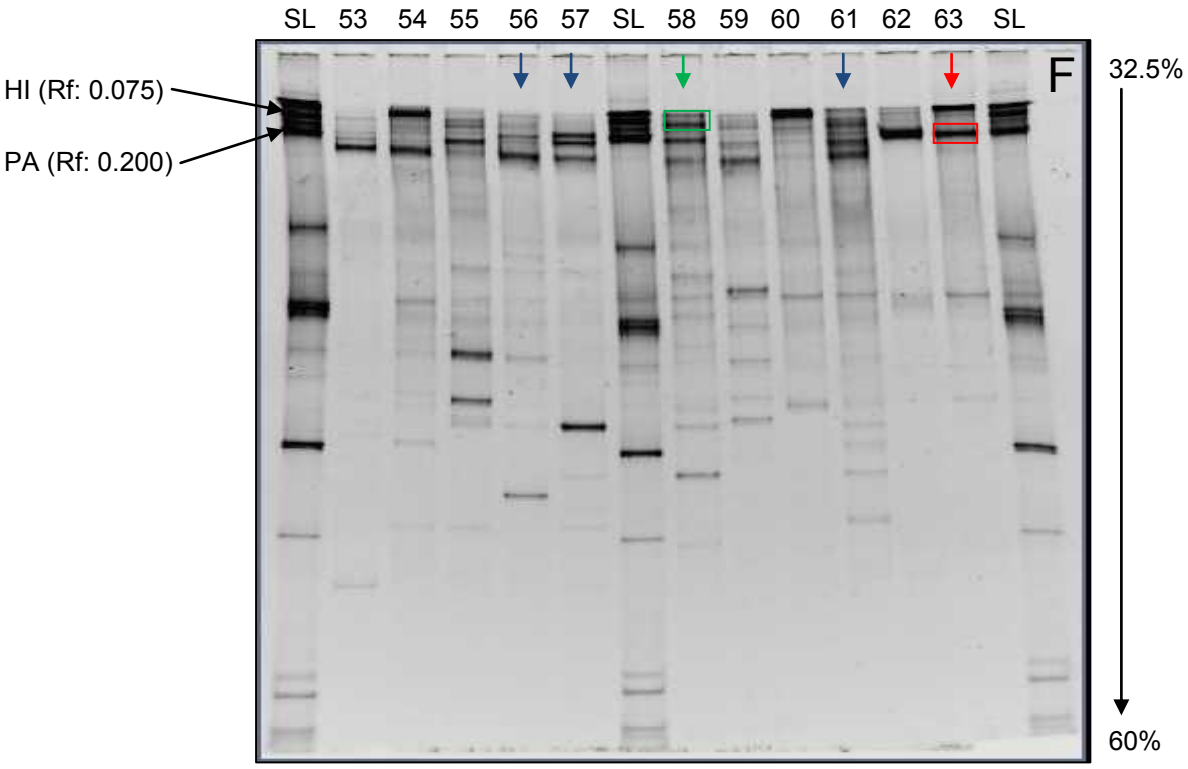
Fig. 4.1A-G: DGGE profiles of whole nCFBR cohort investigated using a 16*S* rRNA PCR-DGGE culture-independent approach. Amplicons were run out on a 32.5-60 % denaturing gradient (shown by arrow). Each band migrated was treated as a single bacterial taxon for the purposes of community profiling and multivariate statistical analysis subsequently carried out. Patient numbers are represented by a sequential series of numbers above each lane. Those lanes denoted with an X (and a red line) represent those individuals removed from the study. Patients indicated with blue, green and red arrows represent patients that are culture-negative and culture-positive for only *H. influenzae* or *P. aeruginosa* respectively. The 16*S* standard ladder (SL) comprised of V3 rDNA fragments derived from pure bacterial cultivars is also shown. Because of the inter-gel variation in the 16*S* SL reproducibility, the Rf values (in parentheses) derived for each bacterial species are only indicated in DGGE profile D for clarification purposes. AX, *Achromobacter xylosoxidans*; BCC, *Burkholderia cepacia* complex; HI, *Haemophilus influenzae*; PA, *Pseudomonas aeruginosa*; RP, *Ralstonia pickettii*; SA, *Staphylococcus aureus* and SM, *Stenotrophomonas maltophilia*. Co-migration of bands against the HI and PA 16*S* SL markers are indicated in green and red rectangles respectively.

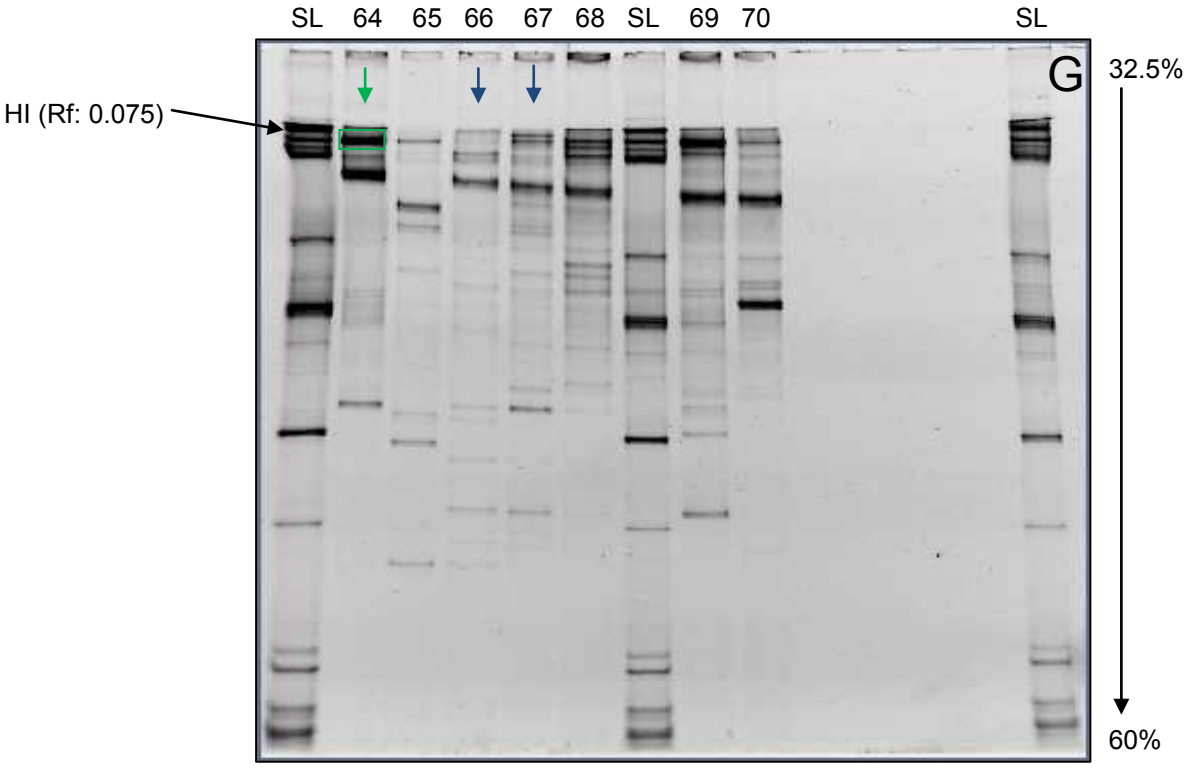

SL 12 13 14 15 16 SL 17 18 19 20 X X SL
B
32.5%
60%
HI (Rf: 0.075)
PA (Rf: 0.200)


SL 21 22 23 24 25 SL 26 X 27 28 29 30 SL
C
32.5%
60%


SL 31 32 33 34 35 SL 36 37 38 39 40 41 SL
D
HI (Rf: 0.075)
SA (Rf: 0.100)
RP (Rf: 0.140)
PA (Rf: 0.200)
BCC/SM
(Rf: 0.400)
AX (Rf: 0.560)
32.5%
60%


SL 42 43 44 45 46 SL 47 48 49 50 51 52 SL
E
32.5%
60%


SL 53 54 55 56 57 SL 58 59 60 61 62 63 SL
HI (Rf: 0.075)
PA (Rf: 0.200)
F
32.5%
60%


SL 64 65 66 67 68 SL 69 70 SL
HI (Rf: 0.075)
G
32.5%
60%

In the DGGE profiles shown in Fig. 4.1A-G, co-migration of bands against the 16*S* standard ladder (SL) annotated strongly suggest the presence of bacterial communities in our nCFBR cohort. However, the 16*S* SL across the DGGE profiles is not strictly reproducible (compare Fig. 4.1D with Fig. 4.1E); therefore any observations noted here are postulated. Culture-negative patients (App. 3) defined by conventional microbiology also included those patients for whom culture of mouth flora was identified (*n* = 19 (27 %)). Multiple bacterial taxa were detected by PCR-DGGE technique across this sub-cohort of patients; these are indicated (Fig. 4.1A-G (blue arrows)). Patient number 21 was reported as culturing for no growth, but detection of bands is present, although these are very faint (Fig. 4.1C, blue arrow and circle).

Culture-positive individuals harbouring *P. aeruginosa* and *H. influenzae* without co-isolation of mouth flora and any other bacterial species numbered eight (11.4 %) and four (5.7 %) patients respectively (App. 3). The co-migration of bands against the annotated HI (Rf 0.075) and PA (Rf 0.200) 16*S* SL markers are shown in these patients (Fig. 4.1A, B, D, E, and F (green arrows and red arrows indicate exclusively HI and PA culture-positive individuals respectively)). In all of the exclusively culture-positive HI individuals' (*n* = 4) co-migration against the HI (Rf 0.075) 16*S* SL marker is confirmed (Fig. 4.1B, D, F, and G (green rectangles)), in addition to other multiple bands being detected. Co-migration of bands against the PA 16*S* SL marker in the exclusively culture-positive PA patients (*n* = 8) is identified (Fig. 4.1A, B, D, and F (red rectangles)) in all but one patient (number 47 (Fig. 4.1E red circle)). As before, detection of numerous bacterial taxa in addition to *P. aeruginosa* is also shown across the DGGE profiles in Fig. 4.1A, B, D, and F. Interestingly, patient numbers 36, 38, 41, and 63 show co-migration of bands against the HI 16*S* SL marker (Rf 0.075). In the culture-based data that was reported co-isolation of *P. aeruginosa* and *H. influenzae* did not occur in the above patients.

Using phenotypic data supplied from the complied cohort's medical database we next investigated the bacterial community profiles generated from DGGE using the Canoco for Windows (v. 4.5.1) and CanoDraw (v. 4.14) software. Analysis of this data was then used to perform an initial detrended correspondence analysis (DCA) giving a 1° axis length of 5.171 (i.e., > 3 SD (standard deviation units of species turnover). Therefore canonical correspondence analysis (CCA (a constrained unimodel ordination method)) was chosen

subjecting the entire cohort (n = 70) with permutation testing under full model (499 permutations) constraining the bacterial community profile variance with 14 measured variables: presence of an exacerbation at sampling (P = 0.002); culture-positive for pathogenic micro-organisms (P = 0.01); isolation of HI and PA from sputum (P = 0.006 and 0.06 respectively); persistent isolation of PA (P = 0.06); intermittent isolation of PA (P = 0.003); no isolation of PA (P = ND (not determined); antibiotic treatment within 1 month of sample submission (P = 0.2); both current colomycin and azithromycin treatment (P = 0.8 and 0.6 respectively) gender (P = 0.2); FEV_1% predicted (P = 0.7); frequent exacerbation (P = 0.9) and finally age (P = 0.6). From the CCA three of the above variables tested for were significant: current exacerbation at time of sampling (P = 0.002), culture-positive isolation of *H. influenzae* (P = 0.006), and intermittent isolation of *P. aeruginosa* (P = 0.003). These variables were significantly associated with the variance of the community structure (Fig. 4.2). Culture-positive identification of *P. aeruginosa* from sputum almost approached significance (P = 0.062).

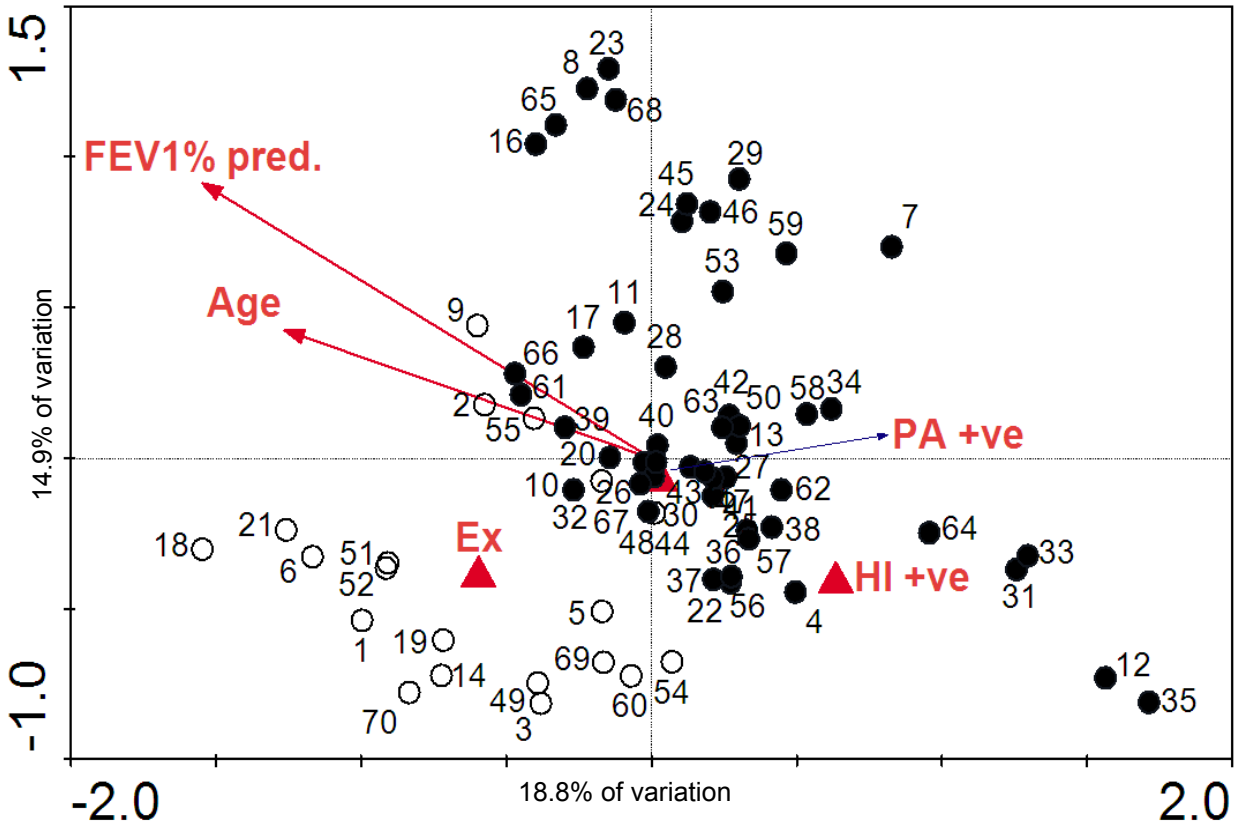


Fig. 4.2: Canonical correspondence analysis of whole patient nCFBR cohort. The ordination plot shows within cohort that the presence of an exacerbation in an individual (*P* = 0.002) and *H. influenzae* colonisation (*P* = 0.006) affects the bacterial community assembly. The first (*x*-axis) and second (*y*-axis) axes explain 18.8 % and 14.9 % of the variance respectively. From the 14 patient phenotypes in the analysis only five are shown for clarity. Symbols represented are: ○, exacerbated bacterial profile individual; ●, stable bacterial profile individual. Discreet variables are indicated by ▲: Ex, exacerbation present; HI +ve, isolation of *H. influenzae* from sputum culture; PA +ve, isolation of *P. aeruginosa* from sputum culture (blue arrow indicates discreet variable). Continuous variables indicated by arrows are FEV_1% predicted and age.

In addition to the whole cohort and the ordination analysis we also investigated a sub-cohort of nCFBR patients who presented with an exacerbation at time of sampling (n = 20). As before, an initial DCA was executed to ascertain the appropriate method of analysing the bacterial community. The primary axis length gradient was 5.918, thus CCA was chosen to perform constrained ordination analyses (Fig. 4.3). From the 13 variables analysed [gender (P = 0.5); age (P = 0.5); FEV_1% predicted (P = 0.6); recent antibiotic treatment within one month (P = 0.2); both current colomycin and azithromycin treatment (P = 0.6 and 0.09 respectively); isolation of PA and HI from sputum (P = 0.7 and 0.6 respectively); no isolation of PA (P = 0.1); intermittent isolation of PA (P = 0.5) and frequent exacerbators within sub-cohort (P = 0.5)] none exerted a significant effect ($P = \leq 0.05$) on the variance explaining the community structure with the exception of those individuals within sub-cohort who were chronically infected with *P. aeruginosa* which approached significance (P = 0.058). As a comparison to the exacerbated individuals, we next examined the stable sub-cohort. As before, an initial DCA was performed (1° axis length gradient = 4.966) followed by a CCA (Fig. 4.4) of the stable sub-cohort resulting in three factors that significantly influenced the bacterial community structure: recent antibiotic treatment (excluding maintenance inhaled colomycin or oral azithromycin administration) (P = 0.04), culture positive detection of PA (P = 0.038), and intermittent colonisation of PA (P = 0.004). Culturable *H. influenzae* was notable (P = 0.08), although within this particular sub-cohort, the isolation from the individuals analysed was small (n = 7). A final constrained ordination analyses was performed on the subset of patients for whom data on exacerbation frequency for the preceding 12 months was available (61 individuals). Although within this sub-cohort of frequently exacerbating patients (n = 38) they did not exhibit a community structure that was significantly different to non-frequently exacerbating patients (data not shown).

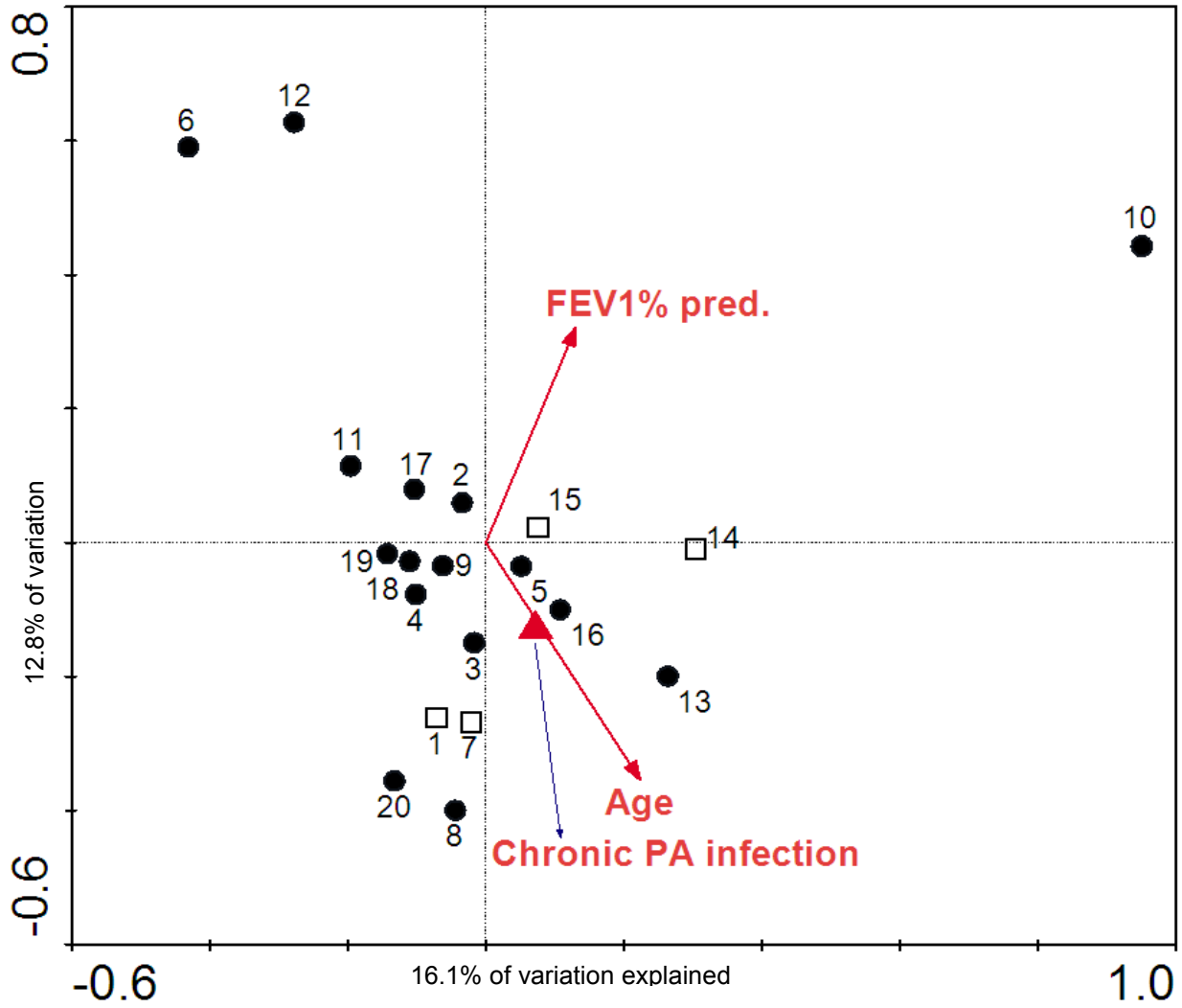


Fig. 4.3: Canonical correspondence analysis of exacerbated nCFBR sub-cohort. Within this sub-cohort only chronic infection with *P. aeruginosa* (PA) approached significance ($P = 0.058$). The first (*x*-axis) and second (*y*-axis) axes explain 16.1 % and 12.8 % of the variance respectively. From the 14 patient phenotypes in the analysis only three are shown for clarity. Symbols represented are: □, individuals with chronic PA infection bacterial profile; ●, individuals with non-chronic PA infection bacterial profile. Discreet variables are indicated by ▲: chronic PA infection (blue arrow indicates discreet variable). Continuous variables indicated by arrows are FEV_1% predicted and age.

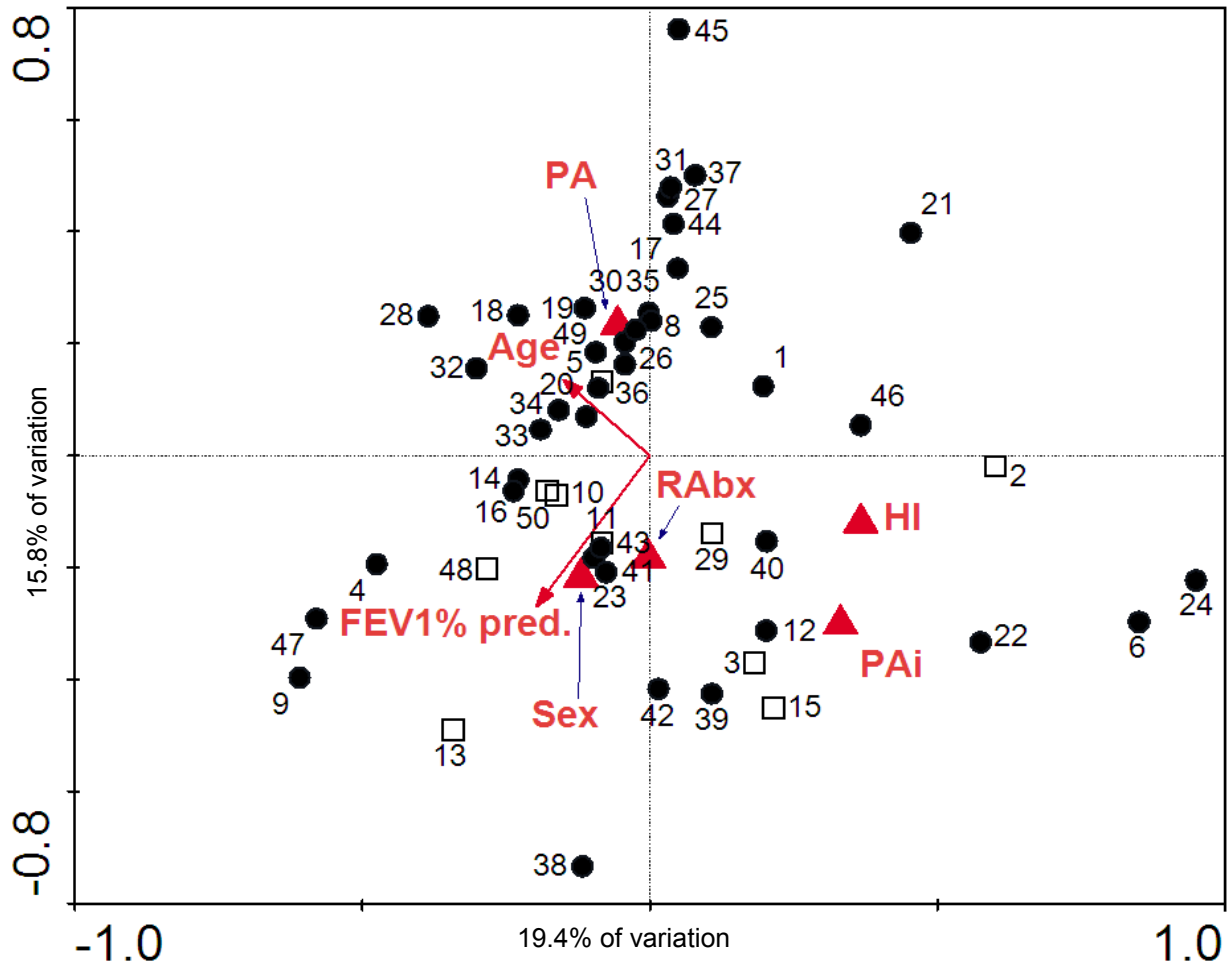


Fig. 4.4: Canonical correspondence analysis of stable nCFBR sub-cohort. Discreet variables indicated by ▲ (blue arrows for clear indication) such as recent antibiotic treatment (RAbx) (P = 0.036), isolation of *P. aeruginosa* (PA) from sputum (P = 0.0038), and intermittent colonisation with *P. aeruginosa* (PAi) (P = 0.004) exerted an effect on the bacterial community structure in sub-cohort. Both gender and isolation of *H. influenzae* (HI) are shown in the CCA output as they were notable from the analysis performed (P = 0.06 and P = 0.08 respectively). The first (x-axis) and second (y-axis) axes explain 19.4 % and 15.8 % of the variance respectively. Symbols represented are: □, individuals with recent antibiotic treatment bacterial profile; ●, non-treatment of antibiotics in patient bacterial profile. Continuous variables indicated by arrows are FEV_1% predicted and age.

4.3.4 Fungal DGGE analysis

From the DGGE analysis performed on whole cohort of the fungal community 1 distinct band position was produced in two sputum samples, one each in patients 50 and 65. The band was compared with a standard ladder of pure fungal isolates and co-migrated with the band corresponding to *Candida glabrata*.

4.3.5 Bacterial community assembly

A total of 2,450 Raup-Crick pair-wise comparisons were carried out and the analysis found that 71 (3 %) of pair-wise comparisons yielded statistically different ($P = \leq 0.05$) communities than would be expected by chance; 225 (9 %) of pair-wise comparisons were statistically similar ($P = \geq 0.95$) and the remaining 2,154 (88 %) had no statistical significance ($P = 0.05 \geq \leq 0.95$).

4.4 Discussion

We have demonstrated three substantive findings from the data presented. Firstly, that the bacterial community profiles from exacerbating individuals were significantly different from those of stable patients (Fig 4.1). From the twenty individuals recruited into the study that were exacerbating when sputum was collected, fourteen individuals had not yet been prescribed antibiotics for their symptoms suggesting that the data was not confounded by therapeutic intervention. The remaining six patients were administered recent antibiotics. The impact of exacerbation in nCFBR is in contrast to studies on the lower airway bacterial community in the CF lung which significantly resulted in no change in community composition as a result of these exacerbations (Tunney et al., 2011, Sibley et al., 2008). Both culture-dependent and culture-independent studies in chronic obstructive pulmonary disease (COPD) suggest that acquisition of new strains is highly associated with a risk of a COPD exacerbation (Murphy et al., 2008, Sethi et al., 2002). Further studies are, therefore, needed to define if nCFBR exacerbations are more analogous to COPD exacerbations rather than CF exacerbations.

Secondly, as in previous studies (Angrill et al., 2002) we found 27 % of samples were culture-negative for recognised pathogens. Molecular analyses demonstrated that these samples did contain a bacterial community with on average 7 taxa which was not significantly different from the culture-positive sputum. Measures of community diversity,

species richness and evenness did not indicate that these indices were significantly different between the communities from culture-positive and culture-negative sputum. Moreover, the community structure was also not significantly different between sputum samples that were culture-positive or -negative. The significance of those taxa that are not routinely identified by culture is unknown. Under some circumstances they may be pathogenic (Tunney et al., 2008), or the increased bacterial load they represent may be associated with airway inflammation and decline in lung function (Weinrich and Korsgaard, 2008). These data are important when considering the management of a patient who fails to respond to antibiotic regimens based on sensitivity results of the predominant species with culture data.

Finally, we have also shown that the presence of *H. influenzae* is significantly associated with a distinct microbial community structure (Fig 4.2). In other studies, it is the most common pathogen isolated from the nCFBR lung (Angrill et al., 2002, King et al., 2007, Pasteur et al., 2001) and is particularly prevalent in paediatric patients. However, in our study the most prevalent organism isolated from sputum was *P. aeruginosa*, which is in agreement with a much earlier study (Nicotra et al., 1995), followed by *H. influenzae*. Infection with *H. influenzae* typically follows a pattern of sequential colonisation by different strains of the bacteria (Grimwood, 2011). *H. influenzae* stimulates mucous production and adheres to and damages epithelial cells, there may also be a role for biofilm formation in resisting host clearance from the lung (King, 2011a). A previous study on a cohort of paediatric CF bronchiectasis patients identified that there was an inverse relationship between *P. aeruginosa* and *H. influenzae* in both culture and metagenomic analyses of the microbial communities (Klepac-Ceraj et al., 2010). However, it was unclear whether the inhibition of *H. influenzae* was due to repeated antibiotic exposure, the arrival of *P. aeruginosa*, or indeed both factors. It is striking in our study that *H. influenzae* was never co-isolated from sputum with *P. aeruginosa*, which supports the observations of Klepac-Ceraj et al. (2010). Moreover, *H. influenzae* was present in patients who had been subjected to long term and repeated antibiotic therapy. This suggests a potentially significant relationship between *P. aeruginosa* colonisation and the loss of *H. influenzae* from the LRT. *P. aeruginosa* has been shown to inhibit the growth of *H. influenzae in vitro* (Riley and Hoffman, 1986) and the interaction between these two major pathogens deserves further study.

There has been some evidence to suggest the stratification of patients with nCFBR based on colonisation with *P. aeruginosa*, with those chronically infected showing significantly lower lung function than intermittently or never colonised patients (Davis et al., 2006, King et al., 2007, Martínez-García et al., 2007). Our data supports these finding with a significant reduction in FEV_1 % predicted ($P = < 0.001$) between those persistently versus intermittently colonised ($P = 0.002$) and persistently versus never colonised patients ($P = < 0.001$). The presence of *P. aeruginosa* has been previously linked to reduced diversity in the bacterial communities in the CF lung (Klepac-Ceraj et al., 2010). Bacterial communities isolated from older CF patients are less diverse, more phylogenetically similar and increasingly dominated by *Pseudomonas* than those of younger CF patients (Cox et al., 2010). Within the total nCFBR cohort there was not a significant association between a patient ever having been colonised by *P. aeruginosa* and the bacterial community structure. In the stable cohort ($n = 50$) the bacterial communities which are intermittently colonised with *P. aeruginosa* are significantly different from those never or persistently colonised (Fig. 4.3). In CF, persistent colonisation is associated with mucoid strains of *P. aeruginosa* and those that have acquired mutations enhance their adaptation to the CF lung (Grimwood, 2011). In contrast, these adaptations are not observed in COPD strains with *P. aeruginosa* infections being more intermittent and sporadic (Rakhimova et al., 2009). Currently, the persistence of *P. aeruginosa* infection and its impact on the overall bacterial community in nCFBR is not known. Our data suggests that *P. aeruginosa* infections are less likely to persist than those seen in CF as only 23 of 70 patients showed persistent *P. aeruginosa* infection. It has also been shown that measures of quality of life are significantly lower in patients colonised by *P. aeruginosa* compared to those that were culture-negative or where *H. influenzae* was the predominant pathogen (Wilson et al., 1997). The factors determining persistence require further study as they may allow new therapeutic interventions and a reduction in the morbidity associated with persistent *P. aeruginosa* infection.

In this study, the patient cohort was recruited from an nCFBR out-patients clinic and as a result the administration of different antibiotics to individuals within the cohort may be a confounding factor to these analyses. We identified 25 patients that were not receiving any antibiotics for one month prior to the collection of samples. Ordination analyses showed that these individuals did not have a significantly different bacterial community to those who were receiving antibiotic therapy. Clinical observations show that antibiotics suppress the

symptoms of exacerbations but have limited effects on chronic symptoms (Anwar et al., 2008). This may be because the non-CF community as in the CF lung are spatially heterogeneous. As a result antibiotic treatment may be ineffective against infections in some areas of the lung (Willner et al., 2012). Our data suggests that antibiotics do not cause a significant perturbation of the bacterial community in the lower airway as determined by analysis of expectorated sputum.

The assembly of the bacterial community in the nCFBR lung does not follow strictly niche or neutral processes, Whereas, the Raup-Crick similarity index of the cohort profiles showed that the majority (88 %) of pair wise comparisons between bacterial profiles indicated neutral (random) community assembly as is observed in the CF lung (van der Gast et al., 2011). Ordination analyses demonstrated a significant role of exacerbation and, colonisation by *H. influenzae* on the community structure suggesting that these factors were drivers for a specific niche community to develop. Similar observations have been reported in other microbial systems (Dumbrell et al., 2010) where initial community assembly is by random immigration of taxa. However, as a community structure develops and interacts with the host lung, environmental niches develop that are occupied by species best adapted to them, resulting in highly adaptable species such as *P. aeruginosa* being common components of the lung microbiome. There appears little consistent colonisation by fungal pathogens within this patient cohort, which is in contrast to the CF airway where fungi have been implicated with lung function decline and increased exacerbations (Bouchara et al., 2009, Chotirmall et al., 2010).

Although we have demonstrated that the lower airways of nCFBR patients are dominated by numerous bacterial taxa by a 16*S* rRNA PCR-DGGE strategy, there are still limitations in using this approach. Firstly, the reproducibility of the 16*S* SL across the DGGE profiles generated and their inherent variance (Fig. 4.1A-G) between these profiles means that the assignment of any bacterial taxa must be postulated. This is additionally confounded by the lack of sequencing data to support our claims when comparing the co-migration of bands in individual patients against the 16*S* SL markers. Indeed, it has been previously demonstrated that PCR fragments from different bacterial species can also migrate at the same rate confounding bacterial species identification even further (Jackson et al., 2000). Other caveats in using 16*S* rRNA PCR-DGGE technique to analyse microbial communities also include

heterogeneity in the 16*S* rRNA gene itself which can often lead to an overestimation of the bacterial community being investigated (Dahllöf et al., 2000, Nübel et al., 1996). Another problem is that the microbial population in using PCR-DGGE is misrepresented or biased because of the detection limits in using the technique, i.e., the rDNA amplicons generated only represent the dominant members present in the community (Muyzer and Smalla, 1998).

Analysis of sputa using conventional microbiology resulted in either *H. influenzae* or *P. aeruginosa* being isolated; none were co-isolated in our whole patient cohort. However, using PCR-DGGE we have revealed that in four patients (Fig. 4.1D and F (numbers 36, 38, 41, and 63)) who were exclusively culture-positive for *P. aeruginosa*, co-migration of bands corresponding to the HI 16*S* SL marker were identified. This identification of *H. influenzae* is postulated as comparing bands against a 16*S* SL is limited by several factors, such as the above. Additionally, the identification of these co-migrated bands could also be assigned either *Haemophilus parainfluenzae* or *Haemophilus haemolyticus*. However, without any sequencing data available from the multitude of bands in each patient presented here, the identification of *H. influenzae* and indeed any other bacterial taxon is presumptive at best. Previous studies have demonstrated that even with molecular phylotyping techniques, the assignment of species within the *Haemophilus* genus itself is difficult (Hedegaard et al., 2001, Nørskov-Lauritsen et al., 2009). An additional culture-independent approach which would have supplemented the data from our PCR-DGGE analysis would have been to employ a deep sequencing strategy via 454-pyrosequencing.

Using conventional microbiology resulted in the detection of 14 bacterial taxa (12 taxa were identified to species level, the two remaining taxa identified to genus level) across our patient cohort. However, in each patient the maximum number of bacterial taxa identified was only two species. Selective media in diagnostic microbiology laboratories is a mitigating factor in this. The use of artificial media is limited in that only 1 % of bacteria can be cultured *in vitro* (Amman et al., 1995, Rappé and Giovannoni, 2003, Staley and Konopka, 1985) this is in addition that several human diseases are now being increasingly recognised as being polymicrobial in nature. Nevertheless, the employment of selective media is still very useful for the determination of bacterial species morphotypes such as the mucoid phenotype in *P. aeruginosa* which is frequently observed in chronically infected CF patients (Foweraker, 2009).

In the past decade it has been previously shown that using culture-independent techniques to investigate other chronic pulmonary diseases that LRT infections could be polymicrobial in nature, as seen in CF (Bittar et al., 2008, Harris et al., 2007, Rogers et al., 2005a, Rogers et al., 2004, Rogers et al., 2003) and increasingly in (COPD) (Cabrera-Rubio et al., 2012, Erb-Downward et al., 2011, Hilty et al., 2010, Huang et al., 2010, Pragman et al., 2012, Sze et al., 2012). Clinical microbiology studies in nCFBR executed a conventional microbiological approach using sputum as a diagnostic marker of bacterial infection. Presently, no studies exist that have investigated the lung microbiome of non-cystic fibrosis patients employing metagenomic culture-independent techniques Recently, a study by Maughan *et al*., (2012) profiled lung tissue samples in both nCFBR (n = 10) and CF (n = 21) patients by a 16*S* rRNA clone library approach. They demonstrated a significant bacterial diversity in the bronchiectasis patients analysed, 955 16*S* rRNA gene sequences were obtained respectively, pertaining to 20 genera being identified in the bronchiectasis sub-cohort alone. Additionally, the most prevalent genus was that of *Pseudomonas* which was present in all ten bronchiectasis patients profiled. Interestingly, *H. influenzae* was detected in only one nCFBR individual. Maughan, *et al*., (2012) suggest that this could be attributable to the sample type analysed; tissue sections from end-stage lung disease as opposed to sputum in earlier nCFBR studies in which the isolation of *H. influenzae* is highly prevalent (Angrill et al., 2002, King et al., 2007, Nicotra et al., 1995, Pasteur et al., 2001). The authors also concluded that no significant differences in the community structure between the nCFBR and CF patients were observed (Maughan et al., 2012).

The recent study by Maughan, *et al*., (2012) partly corroborates our culture-independent data, in particular the finding of significant bacterial diversity in their cross-sectional cohort against our conventional microbiology data. However, in the 16*S* rRNA PCR-DGGE methodology we carried out, expectorated sputum was profiled for bacterial community analysis not lung tissue sections. Additionally, a comparison of the bacterial genera that was identified in the above study from our own data cannot be drawn as no sequencing data was available from our own DGGE profiles that were generated in our nCFBR cohort.

4.5 Conclusions

We have demonstrated that the microbial community of the lower airway in nCFBR is dominated by bacterial taxa. Using a 16*S* rRNA PCR-DGGE approach has revealed several putative bacterial taxa in patients who were initially diagnosed as being culture-negative by using conventional microbiology, although these additional bands could represent the microbial flora in the URT. Furthermore, application of this culture-independent technique has enabled us to discern both *H. influenzae* and *P. aeruginosa* taxa identification in four patients who were all exclusively culture-positive for *P. aeruginosa*. This assignment of the above taxa in these patients is postulated as no sequencing data was obtainable from any of the PCR-DGGE profiles generated in conjunction to the limitations of the technique employed.

Patient exacerbations appear to be associated with a significant shift in the bacterial community therefore, identifying the taxa responsible for these changes are a priority as they may help explain the cause of exacerbation events. *H. influenzae,* when present in the lower airway, is associated with a distinct community profile. However, there appears to be a negative correlation between the presence of *P. aeruginosa* and the persistence of *H. influenzae*. Understanding the nature of this interaction is of interest as it may cast light on the persistence of *H. influenzae* in the lower airway and the dynamics of the microbial community as a whole. In agreement with other studies we have demonstrated that *P. aeruginosa* is a significant pathogen in nCFBR. Patient cohorts do appear to be stratifiable on the basis of the presence and persistence of infection by this bacterium which may be relevant to the design of future intervention trials.

Chapter Five: Molecular fingerprinting and metagenomic analysis in a chronic obstructive pulmonary disease cohort

5.1 Abstract

Culture-independent studies of chronic obstructive pulmonary disease (COPD) are only recently coming to light. Little is known about the role of bacterial colonisation in stable COPD populations. Earlier studies used sputum and classical conventional microbiological techniques to characterise infection in COPD patients. Bronchoalveolar lavage (BAL) fluid was taken from the right lower lobe (RLL) from which genomic DNA (gDNA) was extracted. This was then used as template for amplification of 16*S* rRNA and 28*S* rRNA genes. Amplicons were then run out for molecular fingerprinting by denaturing gradient gel electrophoresis (DGGE). Metagenomic analysis of the 16*S* rRNA gene using 454-pyrosequencing was also performed. Both culture-independent techniques and the bacterial taxa identified were then subjected to multivariate statistical analysis. Culture-based detection resulted in four species. The DGGE profiles produced a distinct number of bands in each sample. The co-migration of bands in each BAL sample was compared against a 16*S* and 28*S* standard ladder enabling putative assessment of which taxa were present. Metagenomic analysis generated 1799 unique OTUs (operational taxonomic units) with the dominant genera identified being *Streptococcus*, *Arthrobacter*, and *Staphylococcus* species respectively across patient cohort. Bacterial OTUs resolved to class-level were found to be significantly associated with lung function (P = 0.002), moderate and severe COPD (P = 0.04), and smoking status (P = 0.05) of patient phenotypes in cohort. Using two culture-independent approaches we have characterised a polymicrobial community in eleven clinically stable COPD patients. Against conventional microbial culture, we have demonstrated the detection of a greater number of bacterial taxa and identified potential drivers of community structure within the COPD lung microbiome.

5.2 Background

Chronic obstructive pulmonary disease is a serious and highly debilitating condition which is an increasing global health problem, ranked the sixth most common cause of mortality worldwide in 1990 (Barnes, 2007), by the year 2020 it is predicted to become the third most common cause of death worldwide and fifth in the cause of disability (Lopez and Murray, 1998). COPD is defined as "a disease state characterised by airflow limitation that is not fully reversible. The airflow limitation is usually progressive and associated with an abnormal inflammatory response of the lungs to noxious particles and gases" (Pauwels et al., 2001). Overall, the most determining factor in the cause of COPD is cigarette smoking (> 95 % of cases in developed countries); however, several other risk factors have been proposed: genetic disposition, poor diet, occupational exposure, and air pollution (Barnes et al., 2003). COPD patients commonly exhibit a disparate, overlapping, multi-factorial pathology of the lower respiratory tract (LRT); including chronic bronchitis, emphysema, mucus plugging, asthma, bronchiectasis, and bronchiolitis (Stockley et al., 2009). Chronic illnesses such as cardiovascular disease, depression, osteoporosis, and lung cancer have also been shown to be adjunct risk factors in patients with COPD (Decramer et al., 2008). Lung pathology in COPD patients leads to episodes of acute exacerbations, i.e., increased dyspnoea, cough, sputum volume and purulence. Over time as COPD progresses, these exacerbations both increase in frequency and severity leading to higher rates of morbidity and mortality in patients (Wedzicha and Wilkinson, 2006).

Historically, the cause of exacerbations was hypothesised to be predominantly by tobacco smoke. However, in the past two decades the cellular and pathological mechanisms that occur during bacterial and viral infections in the respiratory tract has been defined more clearly. In light of this knowledge, there is considerable evidence that infection in COPD patients is now the predominant cause of exacerbations (Sethi et al., 2002, Papi et al., 2006, Murphy et al., 2008, Murphy et al., 2005), although the roles that bacterial and viral agents predispose COPD patients to colonisation or infection with initial development of this disease is less clear and still controversial (Huang and Lynch, 2011). Lung structural damage caused by COPD gives rise to disruption of the innate respiratory tract defences causing mucus gland hypertrophy, hypersecretion, mucocilliary escalator disruption, airway antimicrobial peptide down regulation, and impaired alveolar macrophage function, facilitating the adherence of bacteria when they come into contact with the lower epithelial airways (Bourke, 2002, Sethi

et al., 2009). Colonisation of bacteria in the lower airways induces not only chronic infection in some cases, but also elicits an inflammatory response in COPD patients; both of these factors mitigate disease progression. This phenomenon is known as the vicious circle hypothesis proposed several years ago (Murphy and Sethi, 1992).

The role of bacteria in exacerbations has been primarily investigated in COPD using culture-dependent approaches. These methods have led to the routine isolation of four bacterial pathogens, non-typeable *Haemophilus influenzae* (NTHI), *Moraxella catarrhalis*, *Streptococcus pneumoniae*, and *Pseudomonas aeruginosa* (Sethi and Murphy, 2001); acquisition of new bacterial strains relating to these four species isolates has been previously shown to induce exacerbations in COPD patients resulting in notably increased airway inflammation in contrast to those COPD patients whose exacerbations were not associated with a new strain (Sethi et al., 2007). Indeed, bacterial infection in COPD is associated with ~ 50 % of exacerbations with isolation of *H. influenzae* species accounting for 20-30 % of known bacterial isolates in COPD patients (Huang and Lynch, 2011). Other bacterial isolates such as *Staphylococcus aureus*, *Haemophilus parainfluenzae*, *Haemophilus haemolyticus*, and members from the Enterobacteriaceae family have also been detected in COPD but their role in exacerbating the condition of the disease, particularly in stable COPD patients is unlikely (Sethi and Murphy, 2008). The use of culture-independent techniques exploiting the bacterial 16*S* rRNA gene for metagenomic analysis has only recently come to light in COPD (Erb-Downward et al., 2011, Hilty et al., 2010, Huang et al., 2010), unlike that of CF disease where several studies have been previously published (Bittar et al., 2008, Harris et al., 2007, Rogers et al., 2005a, Rogers et al., 2004, Rogers et al., 2003). Publications in COPD using these culture-independent techniques have successfully demonstrated that the lower airways of COPD subjects are composed of a resident microbial community (termed the lung microbiome), critically, said studies have also elucidated that the LRT in healthy individuals is also colonised with bacteria thus challenging the notion that the lower airways are a completely sterile environment. Definitively speaking, the presence of a lung microbiome in healthy individuals must be approached with caution as they are practical challenges in sampling the lower airways in human subjects such as oropharyngeal contamination in addition to the establishment of what actually constitutes a “healthy” individual in terms of pulmonary disease, as some conditions can be sub-clinical in their presentation. These

measures must also be expressed when assessing the lung microbiome in other chronic pulmonary diseases previously mentioned.

As well as bacterial, viral infection is hypothesised to be a major determinant in inducing acute exacerbations in COPD patients especially during the winter season (Wedzicha, 2004). Viruses such as influenza A and B, rhinovirus, and adenovirus are highly prevalent in the community during these months and could be triggers of acute exacerbations in COPD patients. Pulmonary function decline and increases in clinical symptom severity in viral and bacterial co-infection in COPD patients has also been shown in exacerbations thus lengthening hospital bed stay (Papi et al., 2006, Wilkinson et al., 2006). Fungal infection induced acute exacerbation in COPD is currently undefined and their pathogenesis is not yet known, however, associations between the eukaryotic fungal pathogen *Pneumocystis jiroveci* and lower airway colonisation in COPD has been demonstrated (Morris et al., 2004).

This chapter describes a comparison between traditional culture-based detection versus both molecular fingerprinting and a metagenomic approach via DGGE and next generation sequencing provided by 454-pyrosequencing from the eleven clinical samples analysed. Colonisation of bacteria in the lower airways due to the impairment of the innate immune system in the respiratory tract in COPD patients is hypothesised to be major contributing factor for the induction of acute exacerbations. This constant microbe-host agonist autoimmune response plays a major role in the damage of the lung tissue architecture as in the vicious circle hypothesis. The investigation of these colonising bacteria, and indeed viral infection, in COPD is critical for effective prophylactic and antibiotic administration regimen development to treat this increasingly significant disease.

5.3 Results

5.3.1 Patient cohort analysis

From the 11 patients within the cohort (App. 5), they were two females (CS#9 and CS#12) and nine males, of which 3 were current smokes (CS#5, CS#9, and CS#10) the rest ex-smokers (n = 8). Patient CS#11 was unable to submit a BAL sample so bronchial secretions (BS) were produced instead. Mean age (65), height (1.65 m), forced expiratory volume in one second (FEV_1) (1.40), and FEV_1% predicted (50 %) of cohort were also calculated

respectively. The FEV_1% predicted was also used to ascertain how severe the COPD was in each patient according to the GOLD (Global Initiative for Chronic Obstructive Lung Disease) stages of COPD. Patients at time of sampling presented with either GOLD stage II (i.e., moderate COPD clinical diagnosis; FEV_1/FVC (forced vital capacity) < 0.7 L or 50 % ≤ FEV_1 < 80 % predicted (CS#4, CS#7, CS#9, and CS#12)) or GOLD stage III (i.e., severe COPD; FEV_1/FVC < 0.7 L or 30 % ≤ FEV_1 < 50 % predicted (CS#1, CS#3, CS#5, CS#6, CS#8, CS#10, and CS#11)). Two patients were not receiving treatment for their COPD at the time of sampling (CS#4 and CS#9); only one patient (CS#4) had a previous high-resolution computed tomography (HRCT) scan resulting in a diagnosis of emphysema in addition to their COPD condition.

5.3.2 Culture-dependent analysis of clinical samples

All eleven patients enrolled in the study had routine culture and sensitivity testing performed in the Microbiology Department, Freeman Hospital, Newcastle upon Tyne, by Health & Care Professions Council (HCPC) registered Biomedical Scientists on the clinical samples submitted, i.e., 10 BALFs and 1 BS sample. Note that sample numbers do not follow consecutively as COPD subject 2 (CS#2) was later found not to be diagnosed with COPD and was subsequently removed from this study. From the eleven COPD subjects, five were positive for isolation of potentially pathogenic bacterial species, in which four COPD subjects' yielded typical bacterial species associated with COPD exacerbations (CS#1, *S. pneumoniae*; CS#3, *H. influenzae*; CS#8, *M. catarrhalis*, *H. influenzae*; and CS#10, *M. catarrhalis*). Detection of an atypical pathogen in COPD was observed in CS#6 in which growth for *Chryseobacterium indologenes* was positive. The remaining six other COPD subjects yielded culture-negative growth; CS#4, CS#5, CS#7, CS#9, CS#11, and CS#12. Out of the 11 COPD patients, only CS#3 was positive for the growth of the yeast forming *Candida* spp. (species name was undetermined form the culture-based data provided).

5.3.3 Ecological analysis of bacterial and fungal communities by DGGE

Molecular fingerprinting analysis by DGGE of the 11 COPD subjects yielded 23 and 18 distinct bands for the bacterial and fungal community analysed respectively. From the bacterial DGGE profile generated (Fig. 5.1) a mean of 8 bands per lane was deduced (2-17 bands present per lane), whereas in the fungal DGGE profile (Fig. 5.2), band range was less, 2-10 bands per lane, with a mean value of 5 bands per lane. Each individual band from the

DGGE profiles produced was thus assumed to be a single taxon or operational taxonomic unit (OTU) from which measurements of species richness and evenness could be derived. Shannon diversity (*H'*) indices were calculated and gave a mean of 1.39 (range, 0.423-2.28) for bacterial and 1.24 (range, 0.553-2.02) for fungal diversity present across cohort.

Qualitatively, from the bacterial DGGE profile (Fig. 5.1), there is a strong band presence across the cohort towards the top of the gradient (upper 25 % of gel image shown (Fig. 5.1)), in which the bands present correspond to the bacterial organisms *H. influzenae* (Rf 0.075), *S. aureus* (Rf 0.100), *Ralstonia pickettii* (Rf 0.140), and *P. aeruginosa*. Although, these four bands migrated very closely together, the most prominent bands of the four reference organisms is that of *P. aeruginosa* and *H. influenzae*. Co-migration of bands matching that of *P. aeruginosa* was in all COPD subjects with the exception of CS#6. Samples containing bands that co-migrated with *H. influenzae* were present in CS#5, CS#7, CS#8, CS#10, CS#11, and CS#12. Bands that migrated further down the denaturing gradient, i.e., in the middle, are present, although across cohort these tend to be fainter than those produced in the upper 25 % of the denaturant gel. Migration of bacterial taxa at the bottom of the denaturing gradient is shown in only two patients (CS#11 and CS#12). Three COPD subjects (CS#6, CS#11, and CS#12) showed possible co-migration of bacterial taxa corresponding to the atypical cystic fibrosis pathogen *Achromobacter xylosoxidans* (Rf 0.560).

From the fungal DGGE profile (Fig. 5.2), *Aspergillus fumigatus*, *Exophiala dermatitidis*, and *Scedosporium apiospermum* organisms whose bands all co-migrated very closely together (Rf values 0.660, 0.700, and 0.770 respectively), only samples CS#6 (Rf 0.740), CS#8 (Rf 0.700), and CS#12 (Rf 0.740) produced possible bands corresponding to these three fungal organisms. In contrast, four COPD patients, CS#1, CS#6, CS#7, and CS#9 revealed fungal taxa that co-migrated with the yeast-forming species *Candida glabrata* (Rf 0.340). However, in CS#7 a band was identified that co--migrated with *Candida albicans* (Rf 0.420).

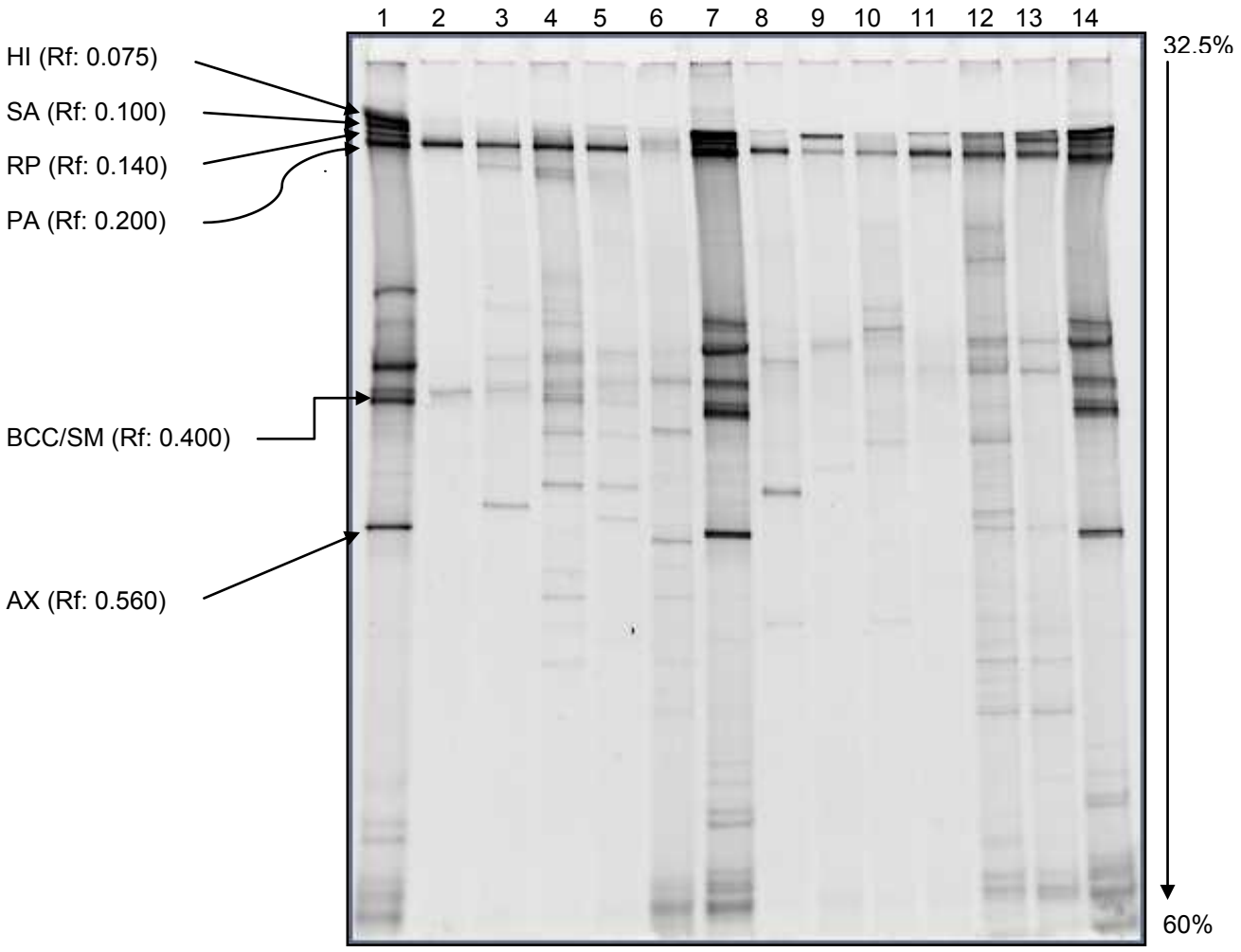


Fig. 5.1: DGGE profile (32.5-60 % denaturing gradient) showing amplified V3 rDNA fragments in COPD cohort. Direction of denaturing gradient and migration of bands during electrophoresis is shown (arrow). Lane numbers: 1, 16*S* standard ladder (SL); 2, CS#1; 3, CS#3; 4, CS#4; 5, CS#5; 6, CS#6; 7, 16*S* SL; 8, CS#7; 9, CS#8; 10, CS#9; 11, CS#10; 12, CS#11; 13, CS#12; 14, 16*S* SL. Rf values of reference organisms in 16*S* SL are also shown indicating migration distances of bands in comparison to distinct bands seen in COPD subjects. AX, *Achromobacter xylosoxidans*; BCC, *Burkholderia cepacia* complex; HI, *Haemophilus influenzae*; PA, *Pseudomonas aeruginosa*; RP, *Ralstonia pickettii*; SA, *Staphylococcus aureus* and SM, *Stenotrophomonas maltophilia*.

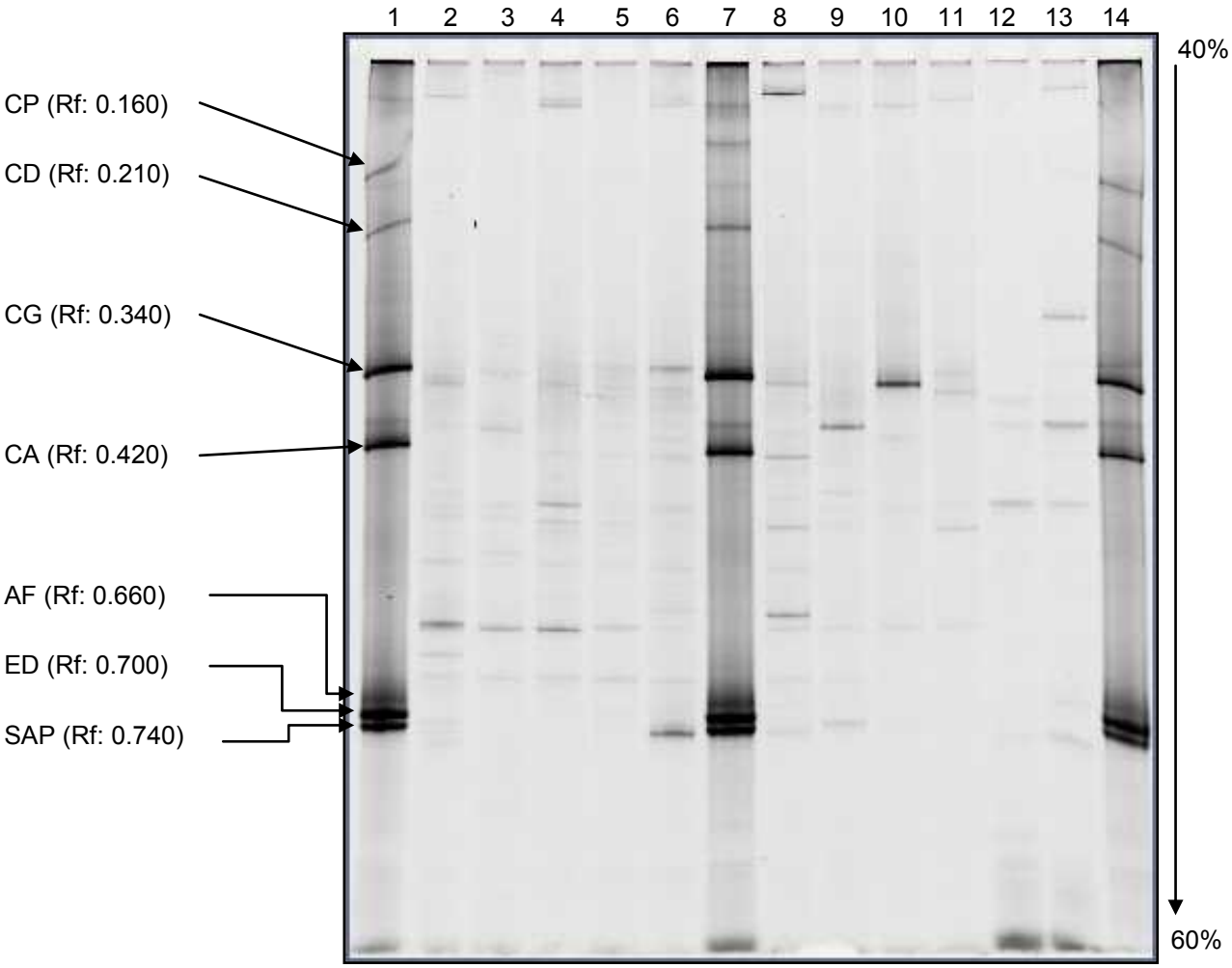


Fig. 5.2: DGGE profile (40-60 % denaturing gradient) showing amplified U1-U2 rDNA amplicons in COPD cohort. Direction of denaturing gradient and migration of bands during electrophoresis is shown (arrow). Lane numbers: 1, 28*S* standard ladder (SL); 2, CS#1; 3, CS#3; 4, CS#4; 5, CS#5; 6, CS#6; 7, 28*S* SL; 8, CS#7; 9, CS#8; 10, CS#9; 11, CS#10; 12, CS#11; 13, CS#12; 14, 28*S* SL. Rf values of reference organisms in 28*S* SL are also shown indicating migration distances of bands in comparison to distinct bands seen in COPD subjects. AF, *Aspergillus fumigatus*; CA, *Candida albicans*; CD, *Candida dubliniensis*; CG, *Candida glabrata*; CP, *Candida parapsilosis*; ED, *Exophiala dermatitidis* and SAP, *Scedosporium apiospermum*.

Ordination plots using Canoco for Windows (v. 4.5.1) of both the bacterial and fungal communities in the COPD cohort analysed were produced. The bacterial community from the DGGE profile generated was analysed by detrended correspondence analysis (DCA) producing a 1° axis length of 2.336 (i.e., < 3 SD (standard deviation units of species turnover) so a redundancy analysis (RDA; a constrained linear ordination method) was executed to analyse the relationship between the bacterial taxa present and environmental variables within cohort at time of sampling. Using Monte Carlo full model permutation testing (499 permutations), analysis of both the contiguous and discreet variables (seven in total including: age (P = 0.612); FEV_1% predicted (P = 0.488); gender (P = 0.374); GOLD COPD stage (P = 0.662); height (P = 0.444) and smoking status (P = 0.274)) against the bacterial community resulted in no significant association between these variables and the variance seen in the community profile. GOLD COPD stages II and III discreet variables were removed from the RDA ordination plot for clarity (Fig. 5.3). The two axes explained 61.5 % and 19.3 % of the variation.

The fungal community analysed by DGGE was also used to generate a second ordination plot in which DCA resulted in a 1° axis length of 3.745 (> 3 SD), therefore canonical correspondence analysis (CCA; a constrained unimodel ordination method) was utilised to investigate the relationship between the fungal taxa and seven environmental variables within cohort as above; age; FEV_1% predicted; gender; GOLD COPD stage; height and smoking status. As before, full model Monte Carlo permutation execution of the fungal community present and the environmental variables resulted in no significant associations between the variance of the community profiles and the discreet and continuous variables (age (P= 0.456); FEV_1% predicted (P = 0.75); gender (P = 0.526); GOLD COPD stage (P = 0.3); height (P = 0.252) and smoking status (P = 0.596)). The first and second axes explained 31.8 % (x-axis) and 26.7 % (y-axis) variation observed in the dataset (Fig. 5.4). As previously, GOLD COPD stages II and III discreet variables were removed in the fungal CCA ordination plot for the purposes of clarity.

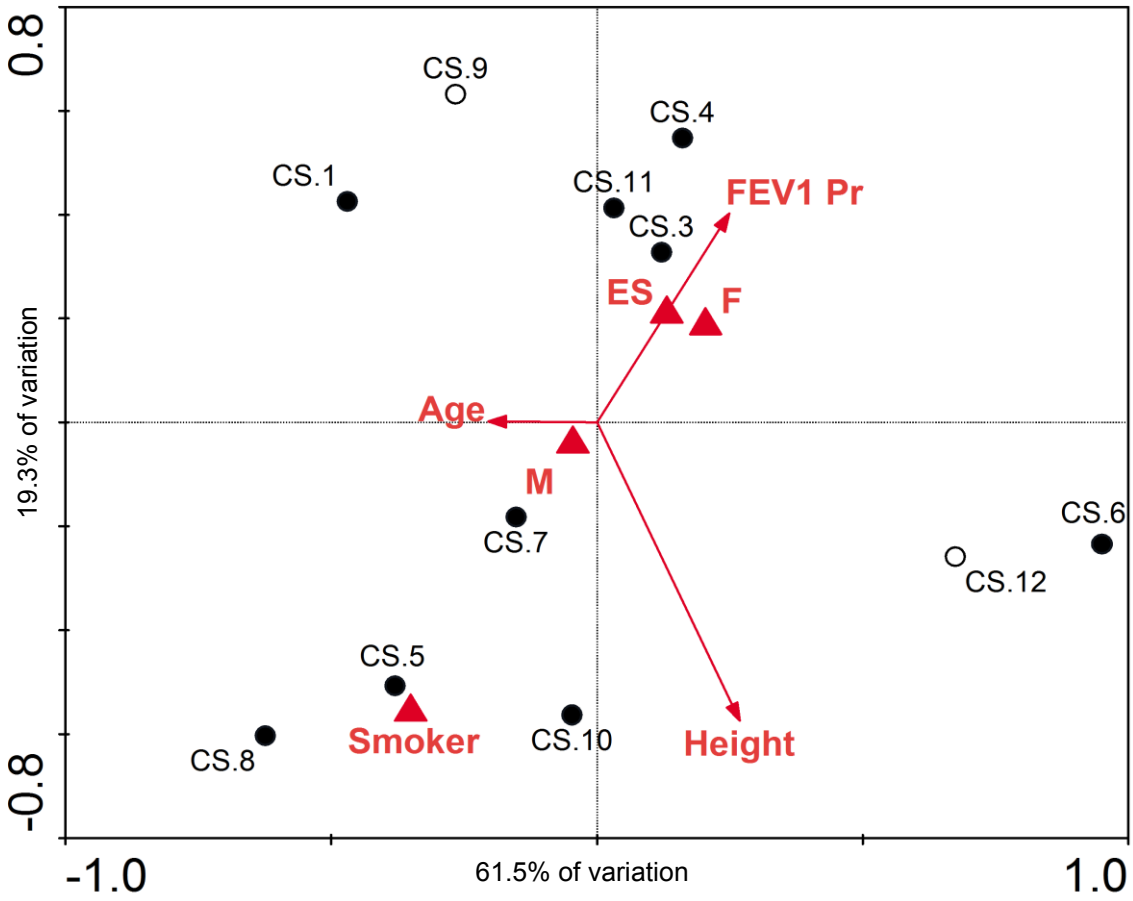


Fig. 5.3: Redundancy analysis showing ordination plot of the bacterial community analysed by DGGE and environmental variables in COPD cohort. An association with the relationship between community structure and environmental variables was not significant. The *x*-axis explains 61.5 % of the variation, whereas the *y*-axis explains 19.3 % respectively. Environmental factors shown consisted of both continuous (height and FEV_1% predicted) and indiscreet variables (smoking status and patient gender). CS.X, COPD subject number (● male; ○ female); ES, ex-smoker; F, female; FEV1 Pr, FEV_1% predicted; M, male.

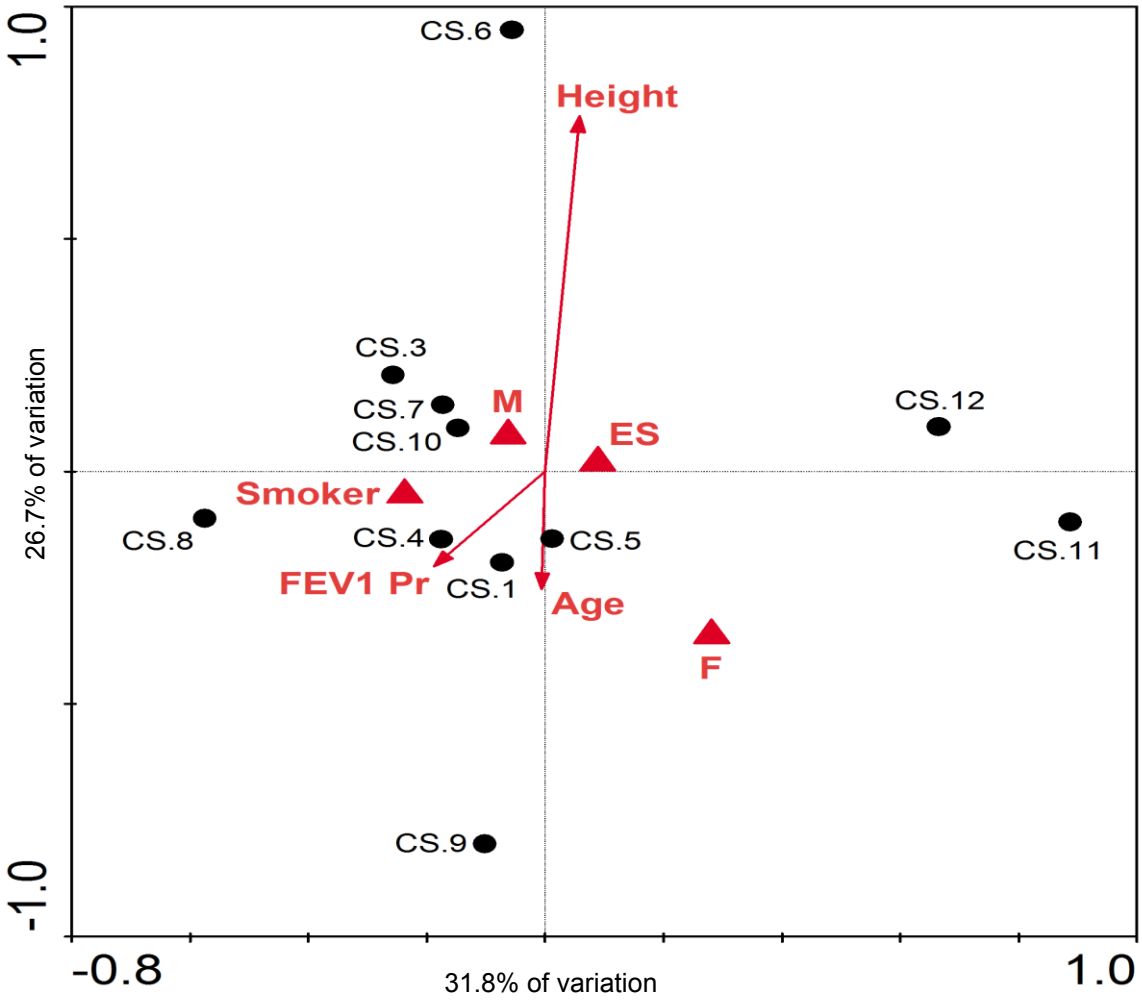


Fig. 5.4: Canonical correspondence analysis showing ordination plot of the fungal community analysed by DGGE and environmental variables in COPD cohort. Associations with the relationship between community structure and environmental variables were not significant. The *x*-axis explains 31.8 % of the variation, whereas the *y*-axis explains 26.7 % respectively. Environmental factors shown consisted of both continuous (height and FEV_1% predicted) and indiscreet variables (smoking status and patient gender). CS.X, COPD subject number (● male; ○ female); ES, ex-smoker; F, female; FEV1 Pr, FEV_1% predicted; M, male.

5.3.4 Metagenomic analysis by 454-pyrosequencing of bacterial community

Analysis of the entire cohort by 454-pyrosequencing from extracted gDNA using the bacterial tag-encoded FLX-Titanium amplicon pyrosequencing (bTEFAP) method created 16*S* rDNA amplicon libraries (Dowd et al., 2008). Following quality control procedures, pyro-amplicons consisting of 380 base pairs (bp) in length were obtained and then assigned to 1,799 unique OTUs generated from the metagenomic data analysis. Following this, a rarefaction curve was generated to ascertain the diversity of the bacterial community present in the clinical samples analysed from the sampling effort (Fig. 5.5). The number of reads per sample and unique number of OTUs assigned are summarised below in table 5.1.

Table 5.1: Number of reads and OTUs assigned per COPD subject from metagenomic analysis.

COPD subject number	Number of reads	Number of unique OTUs assigned
CS#1	2415	504
CS#3	2715	532
CS#4	2308	136
CS#5	3536	447
CS#6	2239	271
CS#7	2657	55
CS#8	1775	126
CS#9	2122	182
CS#10	2148	366
CS#11	542	51
CS#12	557	3

We next investigated the most abundant bacterial genera represented within the community in cohort in rank order. A large number of unique OTUs (1,799) were generated from the 454-pyrosequencing reads therefore any OTUs assigned with < 100 reads per COPD subject were excluded from further analysis. Consequently, assignment of OTUs with > 100 reads was included for further metagenomic analysis of cohort. After this meta-data analysis criterion was met, this resulted in 36 OTUs being designated at genus-level within the cohort,

of which 29 were classified bacterial genera and 7 designated unclassified (Fig. 5.6). Further, from the rankings attributed to the most abundant OTUs, the percentages of these OTUs per COPD subject was determined reflecting the bacterial community at genus level that was present at time of sampling (Fig. 5.7). In terms of abundance, *Streptococcus*, *Arthrobacter*, and *Staphylococcus* were the most prevalent. Strikingly, no OTUs representing the *Pseudomonas* genus were identified within cohort. Investigating individual COPD subjects displayed a heterogeneous bacterial population across the cohort when resolved to genus-level. In samples CS#7, CS#11, and CS#12 the phylogenetic diversity was lower than that seen in the other COPD subjects. Both CS#7 and CS#11 had four bacterial genera present: *Granulicatella Prevotella*, *Streptococcus*, and *Veillonella*. CS#12 was comprised of only two dominant bacterial genera, *Staphylococcus* and *Streptococcus* respectively in the BAL sample analysed. The most abundant genus-level OTUs (i.e., 36) were then resolved to class-level in which 9 different bacterial classes were assigned: Acidobacteria; Actinobacteria; Bacilli; Bacteroidia; Clostridia; α-proteobacteria; β-proteobacteria; γ-proteobacteria and δ-proteobacteria (Fig. 5.8). All COPD subjects were dominated with the Actinobacteria class, with the exception of CS#7, CS#11, and CS#12. These three patients were most prevalent for Bacilli class. The second most dominant bacterial class was that of the α-Proteobacteria, again though, this class was absent in patients CS#7, CS#11, and CS#12.

Ordination analysis using the bacterial community at genus-level was performed. Using the most abundant OTUs generated from the metagenomic analysis (i.e., > 100 reads per OTU designated). An initial DCA was executed to analyse the relationship between the COPD subject bacterial genera in cohort against both discreet and continuous variables resulting in a 1° axis length of 10.776 (axis length > 3 SD). Therefore, canonical correspondence analysis was performed (data not shown as CCA ordination plot is indistinct) resulting in two significant environmental variables: gender (P = 0.026) and height (P = 0.03). Other environmental variables investigated were not significant such as age (P = 0.218), FEV_1% predicted (P = 0.136), GOLD COPD stage (P = 0.076), and smoking status (P = 0.268). Following this, a further investigation of the bacterial community variance at class-level (Fig. 5.9) and their associations with the discreet and continuous variables was conducted in order to ascertain what were the dominant drivers in shaping the COPD community structure. Using CCA (DCA yielded a 1° axis length of 16.969 (> 3 SD)) and the above environmental variables against the nine different bacterial phylotypes represented at

class-level within cohort, two significant factors were identified: FEV_1% predicted (P = 0.002) and GOLD COPD stage (P = 0.04). Smoking status of the COPD subjects, i.e., ex-smoker or current smoker approached significance (P = 0.05). The remaining continuous and discreet variables consisting of age, gender, and height from the CCA output were not significant as P-values of 0.186, 0.132, and 0.14 were all obtained. Both bacterial phylotypes and environmental variables in cohort by CCA (78.4 % (x-axis) and 12.1 % (y-axis) accounted for the percentage of variation explained) are shown in the final output (Fig. 5.9).

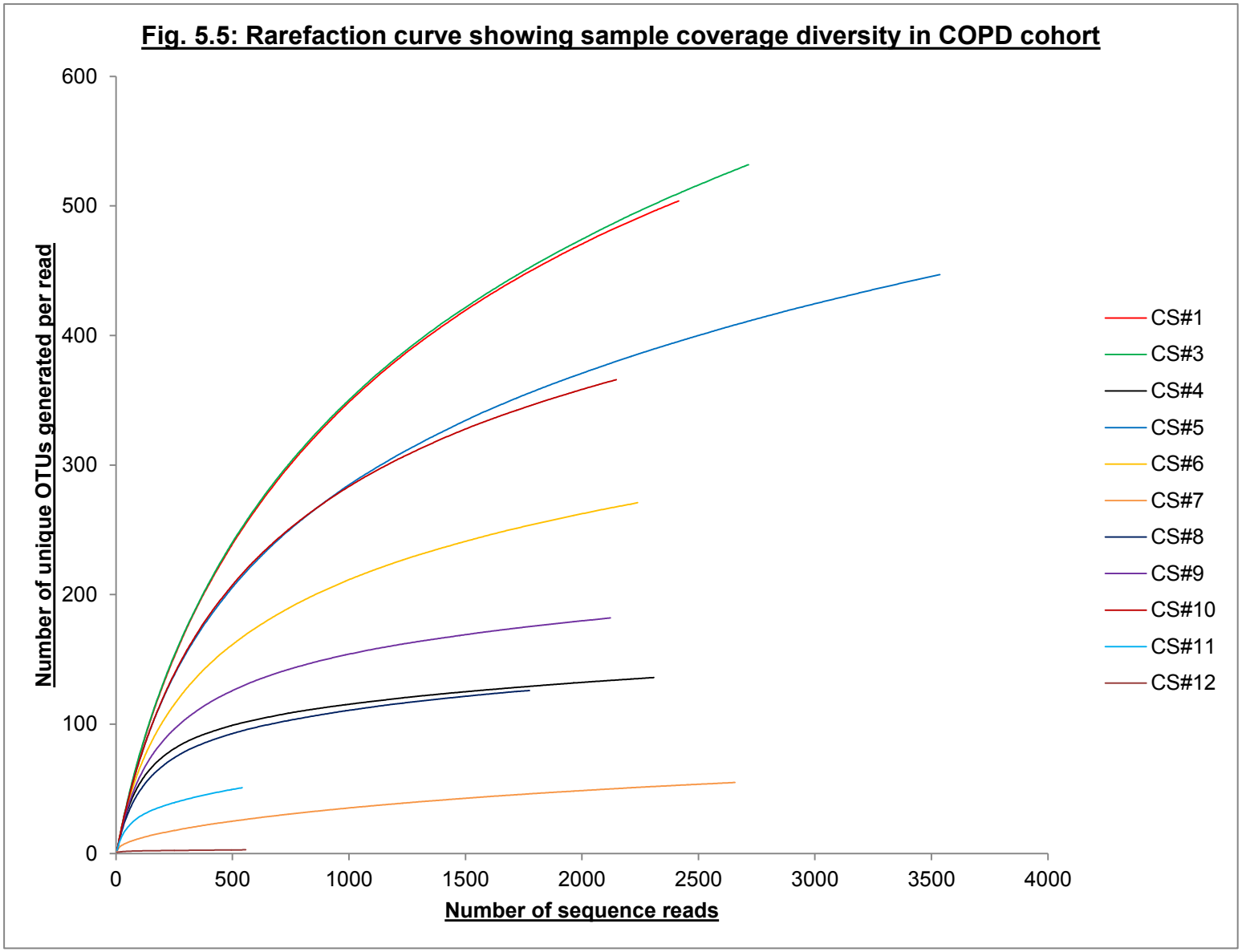


Fig. 5.5: Rarefaction curve showing sample coverage diversity in COPD cohort. Both CS#3 and CS#5 displayed the highest amount of sample diversity, whereas CS#11 and CS#12 exhibited the lowest amount of diversity in their samples analysed.

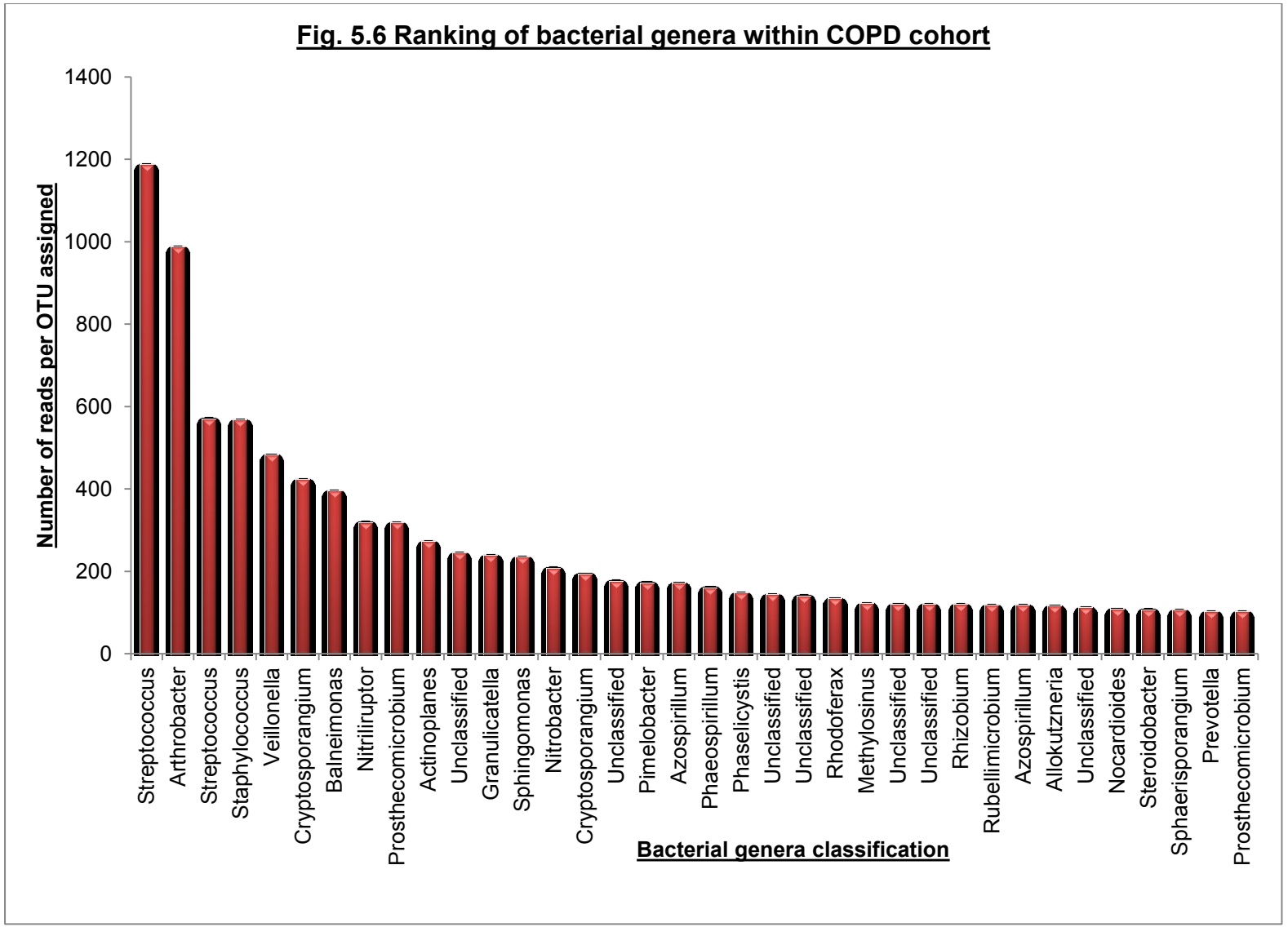


Fig. 5.6: Ranking of bacterial genera within COPD cohort. The most abundant genera across cohort numbered 36 after exclusion of those OTUs that generated < 100 reads per COPD subject. *Streptococcus*, *Arthrobacter*, and *Staphylococcus* were the most abundant bacterial genera present in patient cohort from the 454-pyrosequencing analysis.

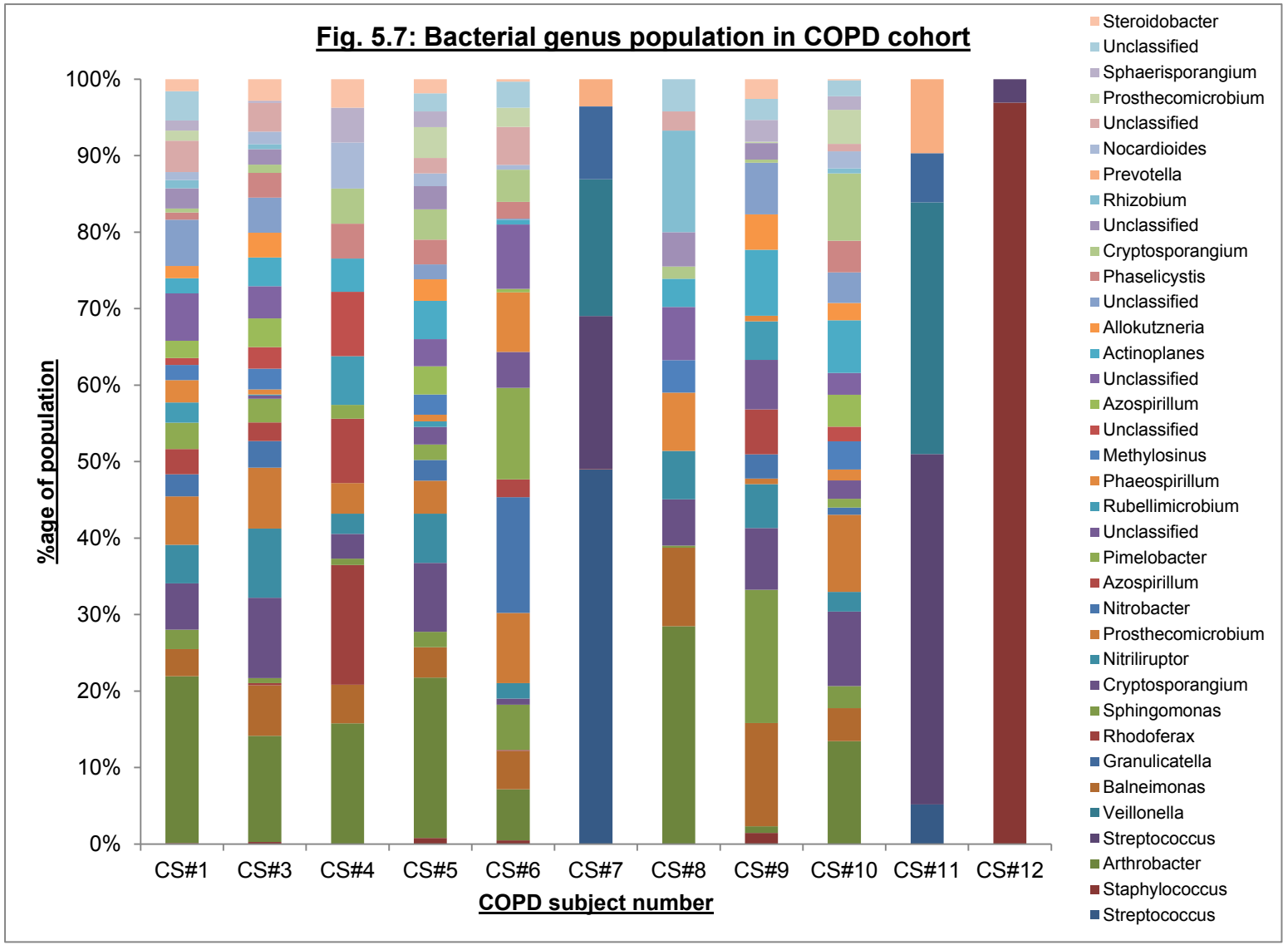


Fig. 5.7: Bacterial genus population in COPD cohort. A percentage population of the bacterial genera composition in the community of each COPD subject analysed from the 454-pyrosequencing data generated. With the exception of CS#7, CS#11, and CS#12, a heterogeneous population is shown in each of the COPD subjects.

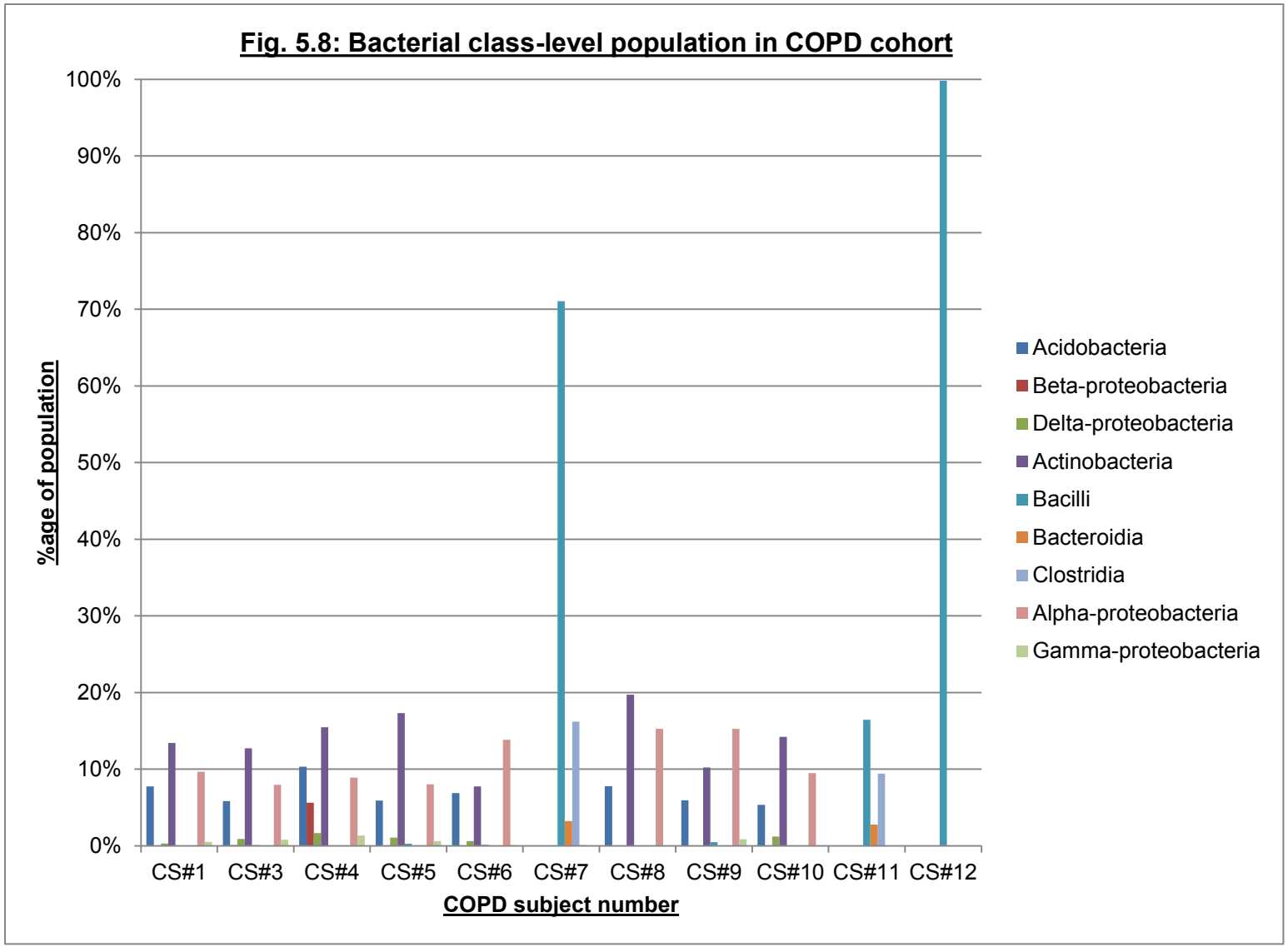


Fig. 5.8: Bacterial class-level population in COPD cohort. Resolution of the 36 most abundant genera at class-level reveals that all of the COPD subjects with the exception of CS#7, CS#11, and CS#12 are populated in bacterial members comprised of the Actinobacteria and α-proteobacteria. The Bacilli class completely dominates CS#12, but also constitutes a significant member of population bacterial class in CS#7 and CS#11.

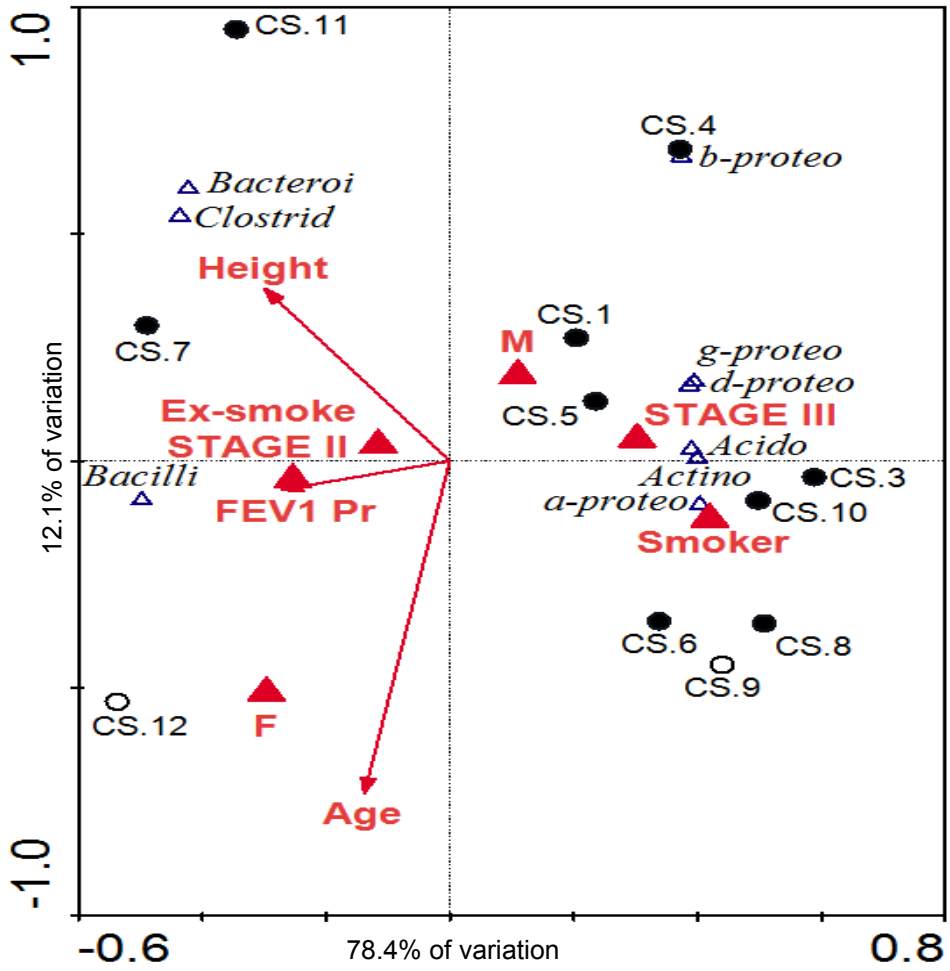


Fig. 5.9: Canonical correspondence analysis of bacterial phylotypes resolved to class-level showing potential drivers of the COPD bacterial community in the COPD subjects analysed. Associations with the relationship between community structure and environmental variables were significant in FEV_1% predicted (P = 0.002), GOLD COPD stage status (P = 0.04), and smoking status in patient (P = 0.05). The x-axis explained 78.4 % of the variation and the y-axis explained 12.1 % of the variation respectively. Environmental factors shown consisted of both continuous (height and FEV_1% predicted) and indiscreet variables (smoking status and patient gender). CS.X, COPD subject number (● male; ○ female); ES, ex-smoker; F, female; FEV1 Pr, FEV_1% predicted; M, male. Bacterial class abbreviations: Acido, Acidobacteria; Actino, Actinobacteria; Bacteroi, Bacteroidia; Clostrid, Clostridia; α-proteo, α-proteobacteria; b-proteo, β-proteobacteria; g-proteo, γ-proteobacteria.

5.4 Discussion

Although patient cohort was small (n = 11), using both a PCR-DGGE mediated approach in conjunction with 454-pyrosequencing metagenomic analysis we have been able to demonstrate several factors in COPD. We have identified the presence of a polymicrobial community in the RLL of the LRT not only comprised of multiple bacterial genera, but strikingly, also perhaps a fungal element. Further, comparison of the bacterial communities identified and their respective populations within each COPD subject demonstrate that they are heterogeneous in nature. Both the bacterial and fungal community generated when a PCR-DGGE culture-independent approach was taken has provided us with a 'snapshot' of the polymicrobial communities exhibited. Both of these community profiles and their respective ordination plots did not reveal any significant factors in the community structure. However, execution of a deep-sequencing strategy and the metagenomic data analysed in addition to the relationship between the bacterial community and patient phenotypes produced significant outcomes in terms of the community structure. The resolution of OTUs from 454-pyrosquencing metadata at two levels, genus- and class-level, with CCA of the bacterial element in COPD subjects where environmental variables within cohort were related yielded varying results; gender and height were significant at bacterial genus-level. Resolution at class-level however revealed that lung function (FEV_1% predicted) and either moderate or severe COPD progression were seen as significant in conjunction with smoking status of individuals.

5.4.1 Bacterial community analysis in COPD cohort by PCR-DGGE

From the PCR-DGGE bacterial community of all COPD subjects some qualitative observations can be made. Intense banding in the upper section of the gel at the top of the gradient suggests that the lungs of the COPD subjects are heavily colonised with bacteria that have a low GC-ratio genomic content such as *H. influenzae*, *M. catarrhalis*, *P. aeruginosa*, and *S. pneumoniae* (Fig. 5.1). Indeed, this has previously been shown in patients suffering from acute exacerbations in COPD as these same bacterial species were isolated from positive culture from expectorated sputa in 59 patients (Larsen et al., 2009). However, in this cohort, sampling strategy involved lower airway BAL from the RLL, not expectorated sputum. Additionally, our patient cohort on presentation at clinic was free of the symptoms associated with those of acute exacerbations.

It has been hypothesised that initial colonisation of pathogenic organisms in patients with COPD in the lower airways elicits an immune response to the pathogen in the host whilst inducing an acute exacerbation. However, acquisition of the same bacterial strain at a later encounter induces a less frequent and severe exacerbation strongly indicating that despite the impairment of the lung innate immune defences, there is still an adaptive immune response in play mediating partial protection against respiratory pathogens (Sethi et al., 2004). A study recently published, described that the lower airways in healthy individuals, i.e., control subjects in their patient cohorts respectively, were not sterile, challenging the bronchial tree sterility hypothesis (Hilty et al., 2010) reported by other authors (Stockley, 1998, Wilson et al., 1996). Hilty, et al., (2010) also analysed the microbial communities in 5 COPD patients, in which pathogenic Proteobacteria, particularly *Haemophilus* spp. were highly prevalent (Hilty et al., 2010). The sampling strategy adopted by Hilty, *et al*., (2010) utilised disposable sheathed cytology brushes and sampled the left upper and right lower lobes of the subject's lungs allowing for a more extensive analysis of the lower airways. Our preliminary data from the PCR-DGGE profile (Fig. 5.1) would support these findings of a microbial community which is present in the LRT of COPD individuals, in particular low GC-count bacterial species like the above.

Execution of a PCR-DGGE approach in this patient cohort yielded the detection of 23 putative bacterial taxa across the 11 COPD individuals enrolled into this study, with a maximum of 88 bacterial taxa detected in total (Fig. 5.1). The use of DGGE in ascertaining these bacterial taxa are reputable as no sequencing data was obtained from any of the co-migrated bands against the 16*S* standard ladder (SL) in each of the COPD subjects profiled. This is against the observed four bacterial species (*H. influenzae*, *M. catarrhalis*, *S. pneumoniae*, and *C. indologenes*) isolated from the BAL specimens using conventional microbiological techniques. However, despite the greater detection of microbial species using DGGE to investigate the bacterial community in our COPD cohort, this is insignificant to our own metagenomic analysis in which 1,799 unique OTUs were generated from 454-pyrosequencing. This is in addition to several recently culture-independent studies investigating the lower airways of COPD patients in which the number of OTUs and sequence reads was far greater than both the PCR-DGGE and conventional microbiology approach presented here. Hilty, *et al*., (2010) identified ~ 3,000 sequence reads in their study (n = 5), Erb-Downward, *et al*., (2011) averaged ~12,000 sequences per sample (n = 14), Sze,

et al., (2012) identified > 1,400 OTUs (n = 8), and finally, Cabrera-Rubio, *et al*., (2012) generated ~ 1,033 sequences from the 16*S* rRNA gene metagenomic analysis corresponding to ~ 500 bacterial species per sample (n = 6).

Comparing the bacterial organisms in the 16*S* SL against the bacterial taxa present in the DGGE profile (Fig. 5.1) several confounding factors can be demonstrated. In preparing the 16*S* SL via PCR-DGGE of the bacterial isolates, an apparent observation was the heterogeneous nature of the 16*S* rRNA gene leading to multiple bands migrating through the denaturing gradient by an individual bacterial isolate. This finding has already been demonstrated, indicating that many bacterial species possess several copy numbers of the 16*S* rRNA gene which can lead to an overestimation of the bacterial community (Dahllöf et al., 2000, Nübel et al., 1996). Additionally, when using 16*S* rRNA PCR-DGGE technique for microbial community analysis it has also been shown that amplicons from different bacterial species can migrate at the same rate (Jackson et al., 2000). This co-migration of different bacterial species can pose problems for the excision of bands for Sanger sequencing applications — i.e., the retrieval of clean DNA sequences from individual bands (Muyzer and Smalla, 1998). No sequencing data was generated from our cohort using PCR-DGGE technique in both the bacterial and fungal communities investigated. Construction of the 16*S* SL by PCR-DGGE has revealed the above caveats, thus an accurate depiction of the microbial community detected in our COPD cohort is putative at best.

Other drawbacks of using the 16*S* rRNA PCR-DGGE approach adopted here also need to be taken into consideration. The employment of a 'semi-nested' approach for PCR amplification of the V3 region could have led to additional biasing factors in the final DGGE profile produced (Fig. 5.1). Furthermore, the attachment of a GC-clamp to the 5′-end of either the forward or reverse primers tends to lower PCR amplification efficiency in addition to increasing the risk of artefact generation and heteroduplex formation (Ferris and Ward, 1997, Lee et al., 1996, Nocker et al., 2007, Ruano and Kidd, 1992). A critical issue in using PCR-DGGE technique is its resolution, i.e., the maximum number of different DNA fragments in a microbial community that can be separated out in the denaturing gradient (Muyzer and Smalla, 1998). Bacterial populations that constitute ≥ 1 % of the total community can be detected by PCR-DGGE as previously shown (Murray et al., 1996, Muyzer et al., 1993), however, the rDNA amplicons obtained in PCR-DGGE mediated studies only reveals the

predominant species present in the community (Muyzer and Smalla, 1998), thus the total population analysed is misrepresented or biased. Nevertheless, despite these inherent limitations, DGGE is a useful technique in comparing community structural changes in response to perturbations or to identify differences observed when comparing bacterial communities in disparate micro-habitats (van der Gast et al., 2008). This would have been invaluable if the BAL samples from our COPD cohort were followed longitudinally as opposed to a cross-sectional enrolment. To date, no present study using a culture-independent approach has addressed this issue.

Traditional culture-based techniques using bacterial morphology suffer from several drawbacks such as the inability of selective media to isolate fastidious and anaerobic bacteria (Staley and Konopka, 1985), in addition to the difficulty of differentiating colonial morphology on plates after swarming with the mucoid phenotype of *P. aeruginosa* (van Belkum et al., 2000). An over-reliance in routine diagnostic microbiology for assessment of the aetiology of microbial infections in COPD is concerning because of the differentiation between colonisation and infection in the host (Lentino and Lucks, 1987). In spite of these limitations, the conventional microbiology data presented here is strongly corroborated by several culture-dependent lower airway investigations in which both stable and exacerbating COPD patients were examined by using quantitative microbial culture methods on BAL samples (Cabello et al., 1997, Soler et al., 1999, Soler et al., 1998). As well as the established microbial agents associated with COPD exacerbations, the identification of *C. indologenes* in CS#6 is unusual as infection with this organism is rare as it is an environmental microbe (Hsueh et al., 1996). However, in a previous study, *C. indologenes* has been implicated in causing pneumonia in mechanically ventilated patients in a critical care unit (Bonton et al., 1993), but there was no report of any patients enrolled into this study as being diagnosed with COPD. A recent retrospective study has been published in which Chen, *et al*., (2012) investigated 125 reported cases of *C. indologenes* being isolated from clinical samples. From their findings, *C. indologenes* was isolated in 91 cases of pneumonia, of which 19 patients presented with COPD as an underlying disease (Chen et al., 2012). The role of *C. indologenes* in stable and exacerbated COPD patients has not been fully investigated yet.

As a comparison between culture-dependent and –independent methods, a recent study investigated clinically stable CF and COPD patients whose expectorated sputum samples

were then inoculated onto six different media. Using terminal restriction fragment length polymorphism (T-RFLP) profiling technique Rogers, *et al*., (2009) also harvested the bacterial growth on these plates for both RNA and DNA extraction, in addition to RNA and DNA being directly extracted from the same sputum sample. The authors concluded that when comparing conventional microbiology and T-RFLP, the former method was shown to be highly selective for the isolation of a small group of recognised respiratory tract pathogens. The use of T-RFLP from both the RNA and DNA directly harvested, in addition to the RNA and DNA extracted from the bacterial growth on diagnostic media, provided a more comprehensive profile of the bacterial species identified confirming that many bacterial taxa are present in sputum samples and that these may be clinically relevant (Rogers et al., 2009b). From the study conducted by Rogers, *et al*., (2009) and the PCR-DGGE approach we employed against our conventional microbiology data, there is partial corroboration, although between out study and Rogers, *et al*., (2009) there is a distinct difference in the patient sampling methodologies and a lack of sequencing data. Furthermore, unlike that of Rogers, *et al*., (2005) only DNA was extracted from the clinical BAL samples, not RNA. Because of this factor, the profiling of the bacterial community represented in our COPD cohort does not indicate which bacterial taxa are metabolically active. This limitation was addressed in a previous study using extracted RNA to detect the presence of metabolically active bacteria in CF sputum samples using reverse transcribed 16*S* rRNA yielding cDNA amplicons which were analysed by reverse transcription T-RFLP (Rogers et al., 2005b).

The identification of microbial communities has been demonstrated for several years in CF using culture-independent methodologies reported in numerous studies (Rogers et al., 2005b, Rogers et al., 2003, Rogers et al., 2004, Rogers et al., 2006, Harris et al., 2007, Bittar et al., 2008) revealing the potential true number of bacterial isolates in CF infected individuals. The preliminary data generated from this small COPD cohort using a 16*S* rRNA PCR-DGGE methodology suggests that a microbial community in the lower airways is perpetuating host inflammatory responses and provides evidence supporting the vicious circle hypothesis (Murphy and Sethi, 1992). However, further analysis is required to indicate whether the bacterial genera in the lower bronchioles represent a core-microbiota in COPD patients as seen recently in CF (van der Gast et al., 2011). Investigations of this core-microbiota in different respiratory disease states such as COPD, CF and non- CF

bronchiectasis could prove valuable for additional studies in the lower airways of individuals afflicted with these diseases.

5.4.2 Metagenomic analysis of bacterial communities in COPD cohort

As previously shown, we have identified a diverse bacterial community present in the LRT of the RLL in a cohort of eleven COPD subjects. Further, using constrained community ordination analysis we also have demonstrated that specific COPD phenotypic features significantly affect the community structure. Culture-dependent microbiological studies focusing in COPD have long been able to identify four main bacterial species, all thought to induce acute exacerbations in COPD: NTHI, *M. catarrhalis*, *S. pneumoniae*, and *P. aeruginosa* (Sethi and Murphy, 2001). Recently though, investigators using 16*S* rRNA community profiling techniques have revealed that the COPD lung microbiome is composed of a greater community richness than previously hypothesised (Erb-Downward et al., 2011, Hilty et al., 2010, Huang et al., 2010). Comparing these studies against the work performed here, interesting observations can be made. Huang and co-authors investigated a small cohort of eight COPD patients being managed for severe respiratory exacerbations. Using 16*S* rRNA PhyloChip technology on endotracheal aspirate (ETA) samples they were able to define 1,213 bacterial taxa present of which 38 bacterial phyla and 140 distinct families were characterised in the lower airways of the COPD cohort investigated. Among the families detected were Alteromonadaceae, Burkholderiaceae, Comamonadaceae, Enterobacteriaceae, Helicobacteraceae, Pasteurellaceae, and Pseudomonadaceae respectively (Huang et al., 2010). Further, despite all of the COPD subjects enrolled into the study being administered broad-spectrum antimicrobial therapies, the ETAs analysed from the patients harboured an average of 411 bacterial taxa. Although this patient cohort was very selective and small in number, it does confirm our current findings of a microbial community residing in the lower airways even though 16*S* rRNA profiling was determined by different culture-independent techniques. However, the diversity reported between our data and Huang, *et al*., (2010) differs dramatically. This difference can be attributed to the fact that in our cohort, sample extraction was performed on eleven clinically stable COPD patients, i.e., non-exacerbating. In contrast, Huang, *et al*., (2010) demonstrated that from their 16*S* rRNA community profiling analysis, patients were positive for the detection of *P. aeruginosa* (belonging to the bacterial family Pseudomonadaceae) in all subjects enrolled. Conversely, in our cohort the

detection of *P. aeruginosa* was not represented in any of the COPD subjects we analysed in significant numbers.

Acquisition of new strains in *P. aeruginosa* has been previously shown to induce acute exacerbations in COPD individuals (Murphy et al., 2008). Additionally, using lower-airway protected brush sampling procedures, Rosell, *et al*., detected the presence of *P. aeruginosa* in their COPD cohort (Rosell et al., 2005); thus between the Huang COPD patient cohort and our study cohort, the absence of *P. aeruginosa* taxa from our metagenomic analysis could explain the clinically stable state of the COPD subjects we investigated. Another plausible reason for the organism's absence from our own metagenomic analysis can be attributed to insufficient numbers present from the OTUs generated from the number of sequence reads. Strikingly, an absence of the *Haemophilus* genera has also been observed from our cohort. *H. influenzae* has long been implicated in COPD bacterial infections and a critical factor in the induction of acute exacerbations in COPD patients (Sethi and Murphy, 2008). The detection of the *Haemophilus* genera in insignificant numbers from the 454-pyrosequencing analysis could be again attributed to the stable state of cohort when sampling was undertaken. Retrospectively though, *H. influenzae* itself is notorious for persistent colonisation in the lungs of COPD sufferers with selected strains persisting for months to years (Murphy et al., 2004). A common disease phenotype of COPD is chronic bronchitis and colonisation of the lower airways is usually associated with NTHI (Cabello et al., 1997) with new strain acquisition and colonisation of the lungs being a continuous and dynamic process (Samuelson et al., 1995). Indeed, such is the persistence of *H. influenzae* in COPD and other chronic pulmonary diseases, that a previous study investigated the distribution of *H. influenzae* in explanted lung tissues from individuals suffering from end-stage pulmonary diseases including COPD, CF and non-CF bronchiectasis (Möller et al., 1998). *H. influenzae* was detected both in the respiratory epithelium and sub-epithelial layers in the explanted lung tissues analysed from the pulmonary end-stage disease patients. This finding strongly suggests that *H. influenzae* plays an invasive role, but also persists in patients with severe lung disease, spreading throughout the pulmonary tree especially in COPD and CF patients (Möller et al., 1998). Additional previous studies have also shown that multiple strains of NTHI can colonise the respiratory tract in concert in chronic bronchitis settings (Murphy et al., 1999, Groeneveld et al., 1988). Excluding *H. influenzae*, both *H. parainfluenzae* and *H.*

haemolyticus are believed to play a role in COPD bacterial infection but their roles in acute exacerbations is uncertain (Sethi and Murphy, 2008).

In another study of COPD subjects, Erb-Downward, *et al*., (2011) investigated the lung microbiome by using massively parallel 454-pyrosequencing of bacterial 16*S* amplicons from not only collected BAL samples but also in explanted lung tissues. They demonstrated three key findings: (i), that the bacterial lung microbiome is present and diverse, but different from the nasopharynx and oral cavity sites, (ii), that a decrease in pulmonary function is associated with a decrease in bacterial community diversity, i.e., the dominance of *Pseudomonas* spp., and (iii), the micro-anatomic sites sampled and analysed within the lung gave rise to disparate microbial communities between them.

In their study, 14 subjects were analysed (7 'healthy' smokers, 4 individuals with COPD (1 mild, 2 moderate, and 1 severe), and 3 non-smokers with no smoking history or evidence of lung disease) and from the metagenomic analysis performed in this cohort, the dominant phyla in the COPD subjects were the Bacteroidetes, Firmicutes, and Proteobacteria. At genus-level, the dominant bacterial genera in the COPD subjects were *Pseudomonas*, *Streptococcus*, *Prevotella*, *Fusobacterium*, and *Veillonella* respectively (Erb-Downward et al., 2011). In contrast, our meta-analysis demonstrated that the predominant bacterial genera were *Streptococcus*, *Arthrobacter*, and *Staphylococcus* indicating a very heterogeneous distribution of microbial diversity across COPD patients. However, in three of our COPD subjects, CS#7, CS#11, and CS#12, the diversity was limited; in particular CS#12 (moderate COPD) only the bacterial genera *Streptococcus* and *Staphylococcus* spp. were detected. This dominance of a very few bacterial genera could be attributed to the one sampling site (RLL) from where the BAL was taken in cohort. Erb-Downward, *et al*., (2011) also demonstrated that sampling from multiple micro-anatomical sites within the lungs demonstrated local differences in the bacterial communities inhabiting them highlighting that bacterial species in the lower airways of COPD patient's exhibit differences not just globally, but also locally.

The observations by Erb-Downward, *et al*., (2011) have only been recently partly corroborated by Sze, *et al*., (2012), although there is still contrasting data presented in their study. The authors of this study investigated the lung tissue microbiome in very severe COPD patients (GOLD stage IV (n = 4)) and compared them to other explanted lung tissues obtained from individuals who were non-smokers (n = 8) and current smoker control subjects

(*n* = 8). Lung tissue samples were also collected from CF patients (*n* = 8) acting as a positive control sub-group — the lungs of CF individuals have been previously shown to contain bacteria and this finding is well documented, both in cross-sectional and temporal studies (Cox et al., 2010, Harris et al., 2007) — for the culture-independent analyses that was carried out in this study.

Executing an approach using terminal-restriction fragment length polymorphism (T-RFLP) and pyrotag sequencing spanning the V1-V3 hypervariable regions within the 16*S* rRNA gene, Sze, *et al*., concluded that the lung tissue microbiome from the COPD GOLD stage 4 patients was significantly different from all of the other lung tissue sections investigated. Strikingly, from their metagenomic analysis in the study both the *Lactobacillus* and *Burkholderia* genera were significantly associated with the COPD GOLD IV explanted lung tissues. Additionally, both of the above genera represented the top five bacterial species within this sample group (i.e., two *Burkholderia* spp. and three *Lactobacillus* spp. were identified, although all were unclassified to species level) (Sze et al., 2012). The bacterial community composition within the GOLD 4 sub-group conflicts with that of the data shown in the Erb-Downward, *et al*., (2011) and Huang, *et al*., (2010) COPD microbiome studies. With our own meta-analysis a comparison cannot be deduced as no GOLD stage IV patients were recruited into our study. However, Sze, *et al*., (2012) state the differences in their study design and COPD patient selection in relation to the two aforementioned studies. They suggest that their investigation reflects the microbiome of lung parenchymal tissue as opposed to the above previous studies which examined the COPD lung bacterial community composition by BAL, ETA, and bronchial brushing collections respectively. Despite some of the conflicting data presented by Sze and co-authors, they conclude that a major limitation in their study was the exclusion of moderate and severe COPD patients (GOLD stages II and III respectively), and as thus, extrapolation of the *Burkholderia* and *Lactobacillus* genera found in their GOLD IV explanted lung tissue samples cannot be made across the other GOLD stages in COPD because of this constraint.

A concerning factor from the metagenomic dataset shown across this patient cohort is the high prevalence of the bacterial genus *Arthrobacter*; in this study *Arthrobacter* spp. was one of the predominant bacterial genera present in all COPD subjects except CS#7, CS#11, and CS#12 (Fig. 5.7). *Arthrobacter* species that are implicated and isolated in clinical human

infection are rare as individuals are continually exposed to *Arthrobacter* strains in the environment (Funke et al., 1996). The first documented cases of *Arthrobacter* infection in patients isolated from clinical specimens by using 16*S* rRNA gene sequencing methods were reported in 1996 resulting in two new *Arthrobacter* species being identified: *Arthrobacter cumminsu* (isolated from a urine sample and skin swab) and *Arthrobacter woluwensis* (isolated from blood culture from a patient presenting with human immunodeficiency virus (HIV) infection) (Funke et al., 1996). Following this, Bernasconi *et al*., (2004) reported a case of infective endocarditis in a patient (HIV-seronegative habitual drug user) in which *A. woluwensis* was subsequently isolated and only identified through full-fragment 16*S* rDNA sequencing (Bernasconi et al., 2004). Initial laboratory diagnosis presumptively identified the organism as *Corynebacterium aquaticum* using conventional microbiology assays. In addition to the Bernasconi case report, there have been only five other documented cases of clinical disease involving *Arthrobacter* spp. none of which involved the pulmonary tree: bacteraemia (2 cases), severe phlebitis (1 case), post-operative endophthalmitis (1 case) and a patient presenting with Whipple's syndrome (1 case) (Bodaghi et al., 1998, Esteban et al., 1996, Hsu et al., 1998, Wauters et al., 2000). The rarity of cases pertaining to the *Arthrobacter* genus can be attributed to its relatively low pathogenicity and the challenges in its precise identification using conventional microbiological techniques such as the above, possibly biasing in a low recovery rate of *Arthrobacter* species (Bernasconi et al., 2004). In our COPD subjects, the dominance of the *Arthrobacter* genus in the metagenomic analysis could be from an environmental source either directly, i.e., *Arthrobacter* and *Corynebacterium* spp. may be a part of the human skin flora, or indirectly. This could be one reason why our metagenomic data-set conflicts with the other COPD lung microbiome studies and as such should be taken into consideration.

In comparing the conventional microbiology data from the COPD cohort to our meta-analysis, the application of a deep-sequencing strategy through 454-pyrosequencing yielded many different bacterial taxa across our COPD patient cohort, in which 1,799 unique OTUs were generated representative to genus-level. Conventional microbiology of patient BAL samples resulted in only four species being isolated: *C. indologenes* (CS#6), *H. influenzae* (CS#3, CS#8), *M. catarrhalis* (CS#8, CS#10), and *S. pneumoniae* (CS#1). Interestingly, from the meta-analysis employed in our study, none of the above genera were identified in our patient cohort from the data shown (Fig. 5.6 and Fig. 5.7) with the exception of the

Streptococcus genus (CS#7, CS#11, and CS#12). Nevertheless, *Chryseobacterium* (CS#11), *Haemophilus* (CS#7, CS#10, and CS#11), and the *Moraxella* (CS#10) genera were present in the COPD subjects analysed in this study (indicated in parentheses), but the assignment of OTUs pertaining to these three particular genera accounted for < 100 sequence reads per OTU. Strikingly, the detection of all the above genera by 454-pyrosequencing conflicts with the conventional microbiology data as *Chryseobacterium*, *Haemophilus*, and *Moraxella* spp. was not detected by culture-based methodologies in CS#7, CS#10, and CS#11 respectively. COPD subject #1 was positive for *S. pneumoniae* growth by culture, but the *Streptococcus* genus itself was not assigned to any OTUs in this individual from the meta-analysis performed. Only the isolation of *M. catarrhalis* by culture in CS#10 corroborates both the culture-dependent and metagenomic analysis performed, as the *Moraxella* genus was also present in CS#10, although the OTU assigned to this genus generated 80 reads.

Historically, from earlier studies, bacterial infection in COPD was established as a potential driver in exacerbations in these patient populations (Burrows and Earle, 1969, Tager and Speizer, 1975). The application of conventional microbiological methods in COPD has primarily identified four organisms highly prevalent in exacerbating COPD patient populations: *H. influenzae*, *M. catarrhalis*, *S. pneumoniae*, and *P. aeruginosa* (Ko et al., 2005, Larsen et al., 2009, Miravitlles et al., 1999, Sethi et al., 2002, Soler et al., 1998). Furthermore, several preliminary studies have been carried out on stable COPD patient cohorts investigating the bronchial tree bacteriologic flora (Brumfit et al., 1957, Lees and McNaught, 1959, Potter et al., 1968). Despite these earlier studies demonstrating the role of bacterial infection and exacerbations in COPD, this paradigm is still controversial. Earlier studies used sputum as a diagnostic measure for determining the role of bacterial infection in COPD (Gump et al., 1976, Lambert and Stern, 1972, McHardy et al., 1980). The execution of sputum as a diagnostic marker coupled with conventional microbiology is limited because of the complexity in the differentiation between colonisation and infection in the individual (Lentino and Lucks, 1987). Furthermore, culture-dependent microbiology of sputum also carries the risk of culturing those organisms that are residents in the URT, thus potentially leading to contamination of the culture plates being analysed.

Comparison and interpretation of the data from the conventional microbiology approach employed here to other studies investigating the respiratory tract flora in stable COPD

patients are difficult because of the differences in microbiological methods and patient selections executed. Some comparisons can be made however from other studies. From our study, culture-dependent microbiology was conducted on BALF samples (with the exception of patient CS#11 in which BS were obtained instead) obtained from the RLL. Additionally, all individuals were stable at presentation before bronchoscopy was performed. The conventional microbiology data is in stark contrast to a study by Cabello, *et al*., (1997) in which they investigated the distal airways of 18 stable non-exacerbating COPD patients by using quantitative culture techniques on both bronchoscopic protected specimen brush (PSB) and BAL samples in the RLL. In their study, Cabello, *et al*., (1997) categorised any bacterial agents identified as either PPMs (potentially pathogenic micro-organisms) or non-PPMs (non-potentially pathogenic micro-organisms. The former being those micro-organisms well established in respiratory tract infections (Gram-positive cocci (*S. aureus* and *S. pneumoniae*); Gram-negative rods (*P. aeruginosa*); Enterobacteriaceae and *Haemophilus* spp; and Gram-negative cocci (*M. catarrhalis*)). The latter comprising of bacterial species such as *Streptococcus viridans* group, *Neisseria* spp., and *Corynebacterium* spp. which are normally associated with either the oropharyngeal or gastrointestinal flora, playing no role in respiratory infection in non-immunocompromised individuals (Cabello et al., 1997).

Cultivation of micro-organisms using both PSB and BAL samples resulted in 22 non-PPMs and 7 PPMs being isolated across 15 COPD patients in their cohort. From the BAL samples analysed, only two COPD patients yielded positive growth, *S. pneumoniae* and *S. viridans*, a PPM and non-PPM respectively. However, 15 COPD patients were positive for bacterial colonisation using PSB technique, resulting in the isolation of 27 micro-organisms, both comprising 5 PPMs (*H. influenzae*, *S. pneumoniae*, and *S. aureus* isolates) and 22 non-PPMs (*S. viridans*, *Neisseria* spp., plasma-co-agulase *S. aureus*, and *Corynebacterium* spp. isolates) (Cabello et al., 1997). In our cohort, five patients yielded positive growth from the BAL samples cultured. Three bacterial species according to the Cabello, *et al*., (1997) categorisation were classified as PPMs: *H. influenzae* (CS#3, CS#8), *M. catarrhalis* (CS#8, CS#10), and *S. pneumoniae* (CS#1). It is also interesting to note that in the Cabello study, both *M. catarrhalis* and *P. aeruginosa* were negative for growth in both BAL and PSB samples analysed, unlike in our COPD cohort in which *M. catarrhalis* was present in CS#8 and CS#10 by culture respectively.

Preceding Cabello, *et al*., (1997) are two earlier studies by Fagon, *et al*., (1990) and Monsó, *et al*., (1995) which both used PSB lower airway sampling technique and quantitative culture to characterise the lower airways in COPD patients. The former study by Fagon, *et al*., (1990) investigated the lower airways of COPD inpatient's mechanically ventilated suffering from severe exacerbations, whereas the latter study by Monsó, *et al*., (1995) sampled the lower pulmonary tree in stable and moderately severe exacerbating COPD outpatients. Evidence for bacterial bronchial pathogens in 50 % of cases was confirmed by both studies using PSB, with the remaining 50 % of cases implying the absence of pathogens (Fagon et al., 1990, Monsó et al., 1995). A comparison in the validity of both study's findings against other techniques such as BAL was not performed by Fagon, *et al*., (1990) and Monsó, *et al*., (1995) as BAL samples from the lower bronchial tree were not analysed in both COPD cohorts enrolled into both studies. Retrospectively, a comparison of our conventional microbiology culture data cannot be made, but both studies corroborate by culture-dependent techniques that the lower airways in COPD patients are colonised both in exacerbated and stable COPD populations. Indeed, other studies have shown that the lungs of stable COPD populations are colonised with bacteria and this has been subsequently demonstrated to contribute towards to the severity and frequency of patient exacerbations (Patel et al., 2002, Rosell et al., 2005, White et al., 2003, Wilkinson et al., 2003).

From their original characterisation of the bronchial COPD pulmonary tree, Soler, *et al*., (1999) investigated 18 control (8 non-smokers and 18 smokers) and 53 stable COPD subjects (28 mild, 11 moderate, and 13 severe COPD phenotypes based on FEV_1) using PSB and BAL sampling techniques. As before, a quantitative conventional microbiology approach was carried out as in the previous studies (Cabello et al., 1997, Soler et al., 1998) in which they found from the PSB and BAL sample analysis, the vast majority of PPMs isolated from both patient cohorts were community acquired, i.e., *S. pneumoniae*, *S. aureus*, *H. influenzae*, and *M. catarrhalis*. Indeed, from the COPD cohort subjected to culture-dependent microbiology, 9 (mild), 11 (moderate), and 2 (severe) COPD patients respectively, was culture-positive for PPMs (Soler et al., 1999). In corroboration with the previous study by Soler, *et al*., (1997), the most prevalent non-PPM isolated was *S. viridans*. The findings by Soler, *et al*., (1999), partly confirm our findings both by culture-dependent and –independent means as they conclusively found that both patient cohorts analysed, in particular their COPD cohort by

BAL sample analyses, were frequently colonised by community acquired PPMs in the bronchial tree (Soler et al., 1999).

The remaining COPD subjects (CS#4, CS#5, CS#7, CS#9, CS#11, and CS#12) in our study were all culture-negative from the conventional microbiology investigation. Although, from the 454-pyrosequencing approach employed, detection of many bacterial taxa was evident. This is clearly apparent in CS#4, CS#5, and CS#9, in which 136, 447, and 182 OTUs pertaining to bacterial genera respectively, were generated from the sequencing reads obtained (Table 5.1). In COPD subjects #7, #11, and #12, in which the number of bacterial genera detected by 454-pyrosequencing was much lower in comparison to the other COPD subjects, the number of OTUs observed is indicative of several bacterial species recovered from the RLL in the BAL samples analysed. Additionally, from the data presented in Fig. 5.7 it is evident that the COPD subjects diagnosed as being culture-negative indeed harbour a lower airway bacterial population varying in diversity.

Furthermore, despite this diagnosis by conventional microbiology, the use of BAL for culture-based microbiological detection is still advantageous over the culture-based methods using sputum as a diagnostic tool, although bronchoscopy itself is still a highly invasive examination technique. The main advantage being that BAL provides an *in situ* representation of the lower airways in healthy or diseased individuals (Reynolds, 2011). However, a previous initial study has demonstrated that recovery of BAL from patients for microbial culture can also yield oropharyngeal commensals; these can sometimes be designated as normal or mouth flora by an individual identifying bacterial species on culture media (Reynolds and Newball, 1974). This identification of normal or mouth flora could have been a biasing factor leading to the conflicting culture-dependent and metagenomic data presented above. The retrieval of BAL however, for the detection of bacterial infection in COPD alongside other investigations is still preferable over sputum due to the decreased risk of contamination from URT organisms in either the oro- or nasopharyngeal cavity.

The use of conventional microbiology for analysis in COPD, and indeed other chronic respiratory diseases such as CF, is also limited in its detection due to the selective nature of the media employed; investigation of the LRT using traditional culture-based methodologies under standard conditions can only culture up to 30 % of bacteria (Suau et al., 1999), this is in addition to culture-dependent techniques being very labour intensive. Also, the

differentiation of bacterial strains isolated from the same species is a confounding factor as these strains cannot be detected by conventional microbiology. Thus detection of different bacterial strains can only be accomplished by molecular typing methods such as pulsed-field electrophoresis. As has been recently shown in the last decade, acquisition of different bacterial strains in COPD is strongly associated with the induction of acute exacerbations in these patient populations (Murphy et al., 2005, Murphy et al., 2007, Sethi et al., 2002, Sethi et al., 2007).

Because of these limitations, many researchers investigating the lower bronchial tree in COPD are now using modern 16*S* rRNA molecular profiling techniques to reveal a bacterial diversity that is greater than previously imagined; this is now being realised (Cabrera-Rubio et al., 2012, Erb-Downward et al., 2011, Hilty et al., 2010, Huang et al., 2010, Pragman et al., 2012, Sze et al., 2012). The preliminary research conducted in CF in the last decade using culture-independent techniques provides a foundation for much of the investigative work carried out in COPD. This approach has resulted in CF now being increasingly recognised as a polymicrobial disease (Rogers et al., 2009a). This is in addition to the complexity of the human oral microbiome and the just recently postulated core respiratory tract microbiome (Avila et al., 2009, Han et al., 2012). Culture-based detection in chronic respiratory tract diseases via expectorated sputum and bronchial washings are still essential for the diagnosis of acute infections or the determination of a carrier state in the patient (Reynolds, 2011). Despite the above limitations, traditional based cultivation of bacterial pathogens still has its advantages though. In CF this is particularly important as conventional microbiological methods enable the detection of the mucoid phenotype established by *P. aeruginosa* in chronically infected CF patients (Fegan et al., 1990, Hassett et al., 2002, Martin et al., 1993, Sokol et al., 1994, Yu et al., 2002). Differentiation between *P. aeruginosa* morphotypes is critical in a diagnostic microbiology laboratory as it has been demonstrated that within these morphotypes there is an inherent variability in their antimicrobial susceptibility patterns (Foweraker et al., 2005), thus this can affect the antibiotic treatment regimen of the CF patients concerned.

5.4.3 Fungal community analysis in COPD cohort by PCR-DGGE

The possibility of a fungal element in stable COPD patients has not been widely reported. Here our analysis revealed a significant and diverse fungal community. This raises several

questions. Does this fungal element contribute towards disease in the lower airways? Do fungi also drive an immunological response; inducing a host-pathogen antagonistic relationship thus contributing towards the vicious-circle hypothesis proposed nearly two decades ago using the bacterial paradigm (Murphy and Sethi, 1992)? Is there an interaction between fungal and bacterial populations that significantly affects disease pathology and severity? Published work on fungal interactions and infections in COPD patients is scarce. A report published several years ago detected the eukaryotic fungal pathogen *Pneumocystis jiroveci* in lung tissues from patients undergoing either lung resection or transplantation in COPD subjects (all GOLD classification stages) (Morris et al., 2004). Aetiology involving *P. jiroveci* infections are generally associated with pneumonia in immunocompromised hosts, specifically, patients infected with HIV (Goldenberg and Price, 2008). However, Morris, *et al* (2004) proposed that colonisation by *P. jiroveci* affected airflow obstruction in smokers, but was greatly pronounced in GOLD IV COPD subjects pointing towards a possible pathogenic link with COPD progression. These findings indicate that in the airways of COPD patients the increasing and irreversible airflow limitation, especially in more severe COPD, provides a more suitable environment for initial colonisation and inevitable proliferation of this fungal pathogen. In our study, FEV_1% was not significant in relation to the fungal community present in the COPD subjects and thus a correlation with reduced airflow obstruction was not shown (Fig. 5.4).

From the culture-dependent microbiology of the COPD subjects, only one patient (CS#3) was positive for fungal growth, identified to genus-level as a *Candida* spp. From the DGGE analysis presented in Fig. 5.2, co-migration of two possible fungal taxa detected in the BAL sample from this patient corresponded to the yeast-forming *Candida* genera, *C. albicans* and *C. glabrata* represented in the 28*S* SL qualitatively. Previous studies in CF individuals have detected numerous fungal species such as *A. fumigatus*, *C. albicans*, *E. dermatitidis*, and *S. apiospermum* (Bouchara et al., 2009, Phiet et al., 2009) some studies support the notion that the above fungal species can be an attributing factor in lung function decline in CF patients (Chotirmall et al., 2010, Haase et al., 1990, Amin et al., 2010, Cimon et al., 2000). However, in terms of COPD disease progression this is currently unproven, and investigations into fungal infections with COPD are unmet. Thus far, only *P. jiroveci* has been implicated in lung function decline in this regard (Morris et al., 2004, Norris and Morris, 2011).

All other environmental variables examined by CCA were not significant although this can be possibly attributed to the small cohort number. In terms of male and female phenotypic relationship to the fungal community only two females were enrolled into this study group, thus having an impact on the constrained community variance analysis performed. Additionally, the detection limits of PCR-DGGE of the fungal community must be considered, even though we have clearly demonstrated that fungal detection in cohort is much greater when using a culture-independent approach as opposed to traditional cultivation methods. Indeed, other culture-independent analysis of potential mycobiomes within diseased and healthy subjects has been previously shown in the oral cavity, but not in the lower airways of COPD patients as stated above. Two recent studies, the first a clone-library approach, investigated the oral cavity in HIV-infected patients using 18*S* rDNA primer sets demonstrating amplification of *C. albicans* and *Saccharomyces cerevisiae* in the subgingival plaque (Aas et al., 2007). The second demonstrated a greater coverage of the oral mycobiome, and thus a more comprehensive profile, by employing a novel Multitag Pyrosequencing approach using the pan-fungal internal transcribed spacer (ITS) regions 1 and 2 nestled between the 18*S* and 28*S* rRNA gene sequences (Ghannoum et al., 2010). From the reads sequenced and subsequently analysed, 101 fungal species were identified, of which 74 genera were culturable and 11 non-culturable in the oral cavity of healthy individuals. Moreover, the number of fungal species inhabiting the oropharynx in these subjects (n = 20) was between 9 and 23 in total with *Candida* being the most frequently isolated genera in 75 % of subjects studied followed by *Cladosporium*, *Aureobasidium*, *Saccharomycetales*, *Aspergillus*, *Fusarium*, and *Cryptococcus* genera respectively (Ghannoum et al., 2010). Although these two studies focused predominantly on the oral cavity, it is interesting to note that the oral cavity contains this much fungal diversity successfully demonstrated by Ghannoum and colleagues, however, against this; a fungal element in the LRT in healthy individuals would be concerning as in nearly all cases of fungal isolation from the lungs points towards an underlying lung pathology, whether it be CF, AIDS in HIV-infected patients or atypical disease phenotypes. Conversely, would it be incorrect to presume that the lung microbiome does indeed contain a fungal element? To date, no metagenomic analysis examining BAL samples from healthy subjects have investigated the potential role fungi may play in the lung microbiome opening the door for other hypotheses.

Critically, the PCR-DGGE approach adopted here is constrained by the choice of primer set and the 'semi-nested' PCR performed. The DGGE profile in Fig. 5.2 representing the fungal community in cohort must be approached, to a degree, with caution. Firstly, the attachment of a GC-clamp to the reverse universal fungal primer U2R-GC may mirror the effects observed in GC-clamp attachment amplicon variation in PCR-DGGE studies of bacterial 16*S* rRNA gene community profiling leading consequently to artefactual production such as chimeras and heteroduplex formation (Muyzer and Smalla, 1998, Rettedal et al., 2010). To counter these effects we used a single batch of primers to offset any potential bias that has been previously reported in using multiple batches of a single primer (Rettedal et al., 2010). Additionally, during the PCR and subsequent amplification of the universal regions within the 28*S* rRNA gene, an additional 30 min during the final elongation step was implemented to further reduce the formation of any artefacts which could adversely affect the fungal community analysis (Janse et al., 2004). Thirdly, because cohort is small and only one clinical sample (either BAL or BS (in the case of CS#11)) was analysed the data at best can only attempt to provide a 'snapshot' of the microbial community identified. Longitudinal studies in this cohort using gDNA from BAL to generate both bacterial and fungal communities via PCR-DGGE profiles would have been complementary to the data presented. Additionally, no sequence data is available from the fungal community.

Retrospectively in terms of a paradigm; fungal infection could have a role in disease progression in COPD subjects in relation to polymicrobial infection processes. Impaired innate lung defence mechanisms in the LRT in COPD induced primarily by smoking in addition to the fungal mycobiome in the oral cavity could predispose COPD patients to fungal infection. The action of smoking on the lower airways has been previously shown to cause defects in mucocilliary clearance thus effecting mucocilliary escalator function, and inducing surfactant abnormalities (Finley and Ladman, 1972, Vastag et al., 1985, Stanley et al., 1986, Verra et al., 1995, Honda et al., 1996). Both of these dysfunctional properties suggestively increase the probability of fungal colonisation in the LRT, as in bacterial colonisation, and in turn decrease the fundamental mechanisms necessary for pathogen clearance.

5.4.4 Microbial community profiles by ordination analysis

Both the 16*S* and 28*S* rRNA PCR-DGGE techniques employed were used to investigate the lower airway microbial community in our COPD cohort. Additionally, the microbial communities detected by the above approach were then subjected to ordination analyses. However, the ordination analyses by the RDA and CCA performed did not show any significant community structure changes in relation to the COPD cohort patient phenotypes analysed (Fig. 5.3 and Fig. 5.4). Conversely, the assignment of OTUs from the metagenomic analysis at two different bacterial phylotypes (representative at class- and genus-level) revealed potential drivers of the bacterial community that we have identified in our COPD cohort by CCA. At class-level, associations with the relationship between community structure and patient phenotypic variables were significant in FEV_1% predicted ($P = 0.002$), GOLD COPD stage status ($P = 0.04$), and smoking status ($P = 0.05$) (Fig. 5.9). At genus-level, both patient gender ($P = 0.026$) and height ($P = 0.03$) phenotypes were shown to be significant in association with community structure (data not shown). These potential drivers of the community structure at genus-level are skewed as only two female individuals were enrolled into our COPD cohort versus nine males. Furthermore, an individual's height and ethnicity can also affect their lung volume respectively (Cotes et al., 2006b, Cotes et al., 2006a, Hughes, 2007). In our COPD cohort database, patient ethnicity was not disclosed for the purposes of this study.

Previous longitudinal studies in CF have shown that in stable patients, there is a decline in FEV_1 with age (Corey et al., 1997, Kerem et al., 1992). Additionally, Cox, *et al*., (2010) investigating an age-stratified CF cohort has shown that a decrease in bacterial diversity and community richness is correlated with an increase in patient age. These findings strongly suggest that bacterial colonisation in CF plays a major role in the frequency and severity of patient exacerbations via lung function decline. Although our cohort was a cross-sectional investigation, from our observations, lung function (FEV_1% predicted) was shown to be significantly associated with bacterial community structure ($P = 0.002$). Two longitudinal studies in COPD have demonstrated lung function decline in stable COPD populations (Marin et al., 2010, Wilkinson et al., 2003). Wilkinson, *et al*., (2003) followed-up 30 stable COPD patients over 1 year and demonstrated that FEV_1 decline was correlated with an increase in bacterial load and species change. Subsequently, Marin, *et al*., (2010) found that colonisation with *H. influenzae*, *P. aeruginosa*, enterobacteria spp. and neutrophilic

inflammatory response markers was significantly associated with a decrease in FEV_1 in a cohort of 40 COPD patients over an eight month period. The studies by Wilkinson, *et al*., (2003) and Marin, *et al*., (2010) not only show interplay between bacteria and lower airway inflammation, but also importantly, that bacterial infection in COPD patients is not a static phenomenon (Beasley et al., 2012). Both the Wilkinson, *et al*., (2003) and Marin, *et al*., (2010) studies partly corroborate our findings in that bacterial colonisation in the LRT exhibits an effect on lung function in COPD individuals.

A potential driver of the microbial community we have identified in our COPD cohort was significantly associated with GOLD stages II and III, i.e., moderate COPD and severe COPD respectively ($P = 0.04$). Chronic obstructive pulmonary disease severity is classified into four stages by spirometric diagnosis which is used to ascertain the severity of pathologic changes occurring in the patients assessed (Rabe et al., 2007). Recently, Pragman, *et al*., (2012) characterised and compared the lung microbiomes of both moderate ($n = 14$) and severe COPD ($n = 8$) patients, including a healthy control group (4 smokers and 6 non-smokers). As in other lung microbiome studies they executed a deep sequencing strategy of the V3 region of 16*S* rDNA amplicons and demonstrated that there was a significant increase in microbial diversity in both the moderate and severe COPD BAL samples analysed in comparison to the healthy control group (Pragman et al., 2012). Furthermore, from the metagenomic analysis performed by Pragman, *et al*., (2012), principle co-ordinates analysis (PCoA) revealed separation between the control and COPD cohorts investigated. However, no separation was observed by PCoA between the moderate and severe COPD BALF samples analysed in this study (Pragman et al., 2012). From our data, there is a clear separation in the patients and their GOLD stage COPD characterisation (i.e., 4 moderate (stage II) and 7 severe (stage III) COPD patients), in addition to the bacterial class phylotypes we have profiled (Fig. 5.9).

In our cohort, eight patients were ex-smokers and three current smokers. These two phenotypic parameters were classified as smoking status in the patient and were significant ($P = 0.05$). A previous study has investigated the microbial communities of the URT (nasopharynx and oropharynx) using a deep sequencing approach comparing smoking ($n = 29$) and non-smoking ($n = 33$) healthy asymptomatic adults (Charlson et al., 2010). Charlson, *et al*., (2010) found global community structure differences in bacterial taxa at both genus and phylum level. The smoker sub-cohort tended to exhibit disordered microbial community

structure than the non-smoking sub-cohort, including an increase in diversity in both the oropharyngeal and nasopharyngeal sites sampled (Charlson et al., 2010). Additionally, the smoker sub-cohort URT microbiome was also characterised by more bacterial genera potentially associated with respiratory tract disease. The data presented by Charlson, *et al*., (2010) is in agreement with our own data suggesting that smoking status in a COPD patient can exhibit an effect on the community structure, although in the above study only the URT was sampled not the LRT.

5.5 Conclusions

The culture-independent techniques against the conventional culture-based methods have enabled us to characterise the lung microbiome in a small cohort of moderate and severe COPD patients. We have also demonstrated that the lower airways in our COPD cohort are composed of a polymicrobial community using both a PCR-DGGE and deep sequencing strategy. Both these bacterial and fungal elements may drive the perpetuating immunological response playing a role in the vicious circle hypothesis. However, no sequencing data was obtained when using a 16*S* and 28*S* PCR-DGGE approach so the profiles we have generated using this technique is putative at best.

The metagenomic data analysed from the 454-pyrosequencing reads has shown that each COPD subject was host to heterogeneous bacterial populations, with the most dominant genera being *Streptococcus*, *Arthrobacter*, and *Staphylococcus* spp. respectively. The deep sequencing approach has enabled us to characterise this COPD cohort to reveal a diverse population in the RLL of the pulmonary tree. The most prevalent bacterial genera we have identified conflicts with other metagenomic studies which have previously profiled the COPD lung microbiome in both stable and exacerbating COPD populations. However, all previous studies executed different sampling strategies involving their COPD cohorts respectively.

From the ordination analyses, both bacterial and fungal communities identified by PCR-DGGE exhibited no significance in terms of patient phenotype and community structure. However, from the metagenomic analysis, the bacterial community identified and resolved to genus-level, both gender and height are shown to be significant in community structure. Resolution at class-level revealed that lung function (FEV_1% predicted), COPD disease

progression (GOLD stage II or III), and smoking status, were all associated with community structure in the cohort analysed.

Molecular-based detection techniques in COPD could point the way towards a greater understanding of the microbial communities in colonised individuals and elucidate the complex mechanisms between host and microbe and their role in the induction of exacerbations.

References

AAS, J. A., BARBUTO, S. M., ALPAGOT, T., OLSEN, I., DEWHIRST, F. E. & PASTER, B. J. 2007. Subgingival plaque microbiota in HIV. *Journal of Clinical Periodontology,* 34**,** 189-195.

AAS, J. A., PASTER, B. J., STOKES, L. N., OLSEN, I. & DEWHIRST, F. E. 2005. Defining the normal bacterial flora of the oral cavity. *Journal of Clinical Microbiology,* 43**,** 5721-5732.

ADÉKAMBI, T., DRANCOURT, M. & RAOULT, D. 2008. The *rpoB* gene as a tool for clinical microbiologists. *Trends in Microbiology,* 17**,** 37-45.

AKINBAMI, L. J., MOORMAN, J. E. & LIU, X. 2011. Asthma prevalence, health care use, and mortality: United States, 2005-2009. *National Health Statistics Reports***,** 1-14.

AKIRA, S., UEMATSU, S. & TAKEUCHI, O. 2006. Pathogen recognition and innate immunity. *Cell,* 124**,** 783-801.

ALEXOPOULOU, L., HOLT, A. C., MEDZHITOV, R. & FLAVELL, R. A. 2001. Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3. *Nature,* 413**,** 732-738.

ALZEER, A. H., MASOOD, M., BASHA, S. J. & SHAIK, S. A. 2007. Survival of bronchiectatic patients with respiratory failure in ICU. *BMC Pulmonary Medicine,* 7**,** 17.

AMERICAN LUNG ASSOCIATION. *Trends in asthma morbidity and mortality* [Online]. Available: http://www.lung.org/lung-disease/asthma/resources/ [Accessed 17th March 2012].

AMIN, R., DUPUIS, A., AARON, S. D. & RATJEN, F. 2010. The effect of chronic infection with *Aspergillus fumigatus* on lung function and hospitalisation in patients with cystic fibrosis. *137*.

AMMAN, R. I., LUDWIG, W. & SCHLEIFER, K. H. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews,* 59**,** 143-169.

ANGRILL, J., AGUSTÍ, C., DE CELIS, R., RAÑÓ, A., GONZALEZ, J., SOLÉ, T., XAUBET, A., RODRIGUEZ-ROISIN, R. & TORRES, A. 2002. Bacterial colonisation in patients with bronchiectasis: Microbiological pattern and risk factors. *Thorax,* 57**,** 15-19.

ANWAR, G. A., BOURKE, S. C., AFOLABI, G., MIDDLETON, P. G., WARD, C. & RUTHERFORD, R. M. 2008. Effects of long-term low-dose azithromycin in patients with non-CF bronchiectasis. *Respiratory Medicine,* 102**,** 1494-1496.

ARMSTRONG, L., MEDFORD, A. R., UPPINGTON, K. M., ROBERSTON, J., WITHERDEN, I. R., TETELY, T. D. & MILLAR, A. B. 2004. Expression of functional Toll-like receptor-2 and -4 on alveolar epithelial cells. *American Journal of Respiratory Cell and Molecular Biology,* 31**,** 241-245.

AVILA, M., OJCIUS, D. M. & YILMAZ, Ö. 2009. The oral microbiota: Living with a permanent guest. *DNA and Cell Biology,* 25**,** 405-411.

BALS, R. & HIEMSTRA, P. S. 2004. Innate immunity in the lung: How epithelial cells fight against respiratory pathogens. *European Respiratory Journal,* 23**,** 327-333.

BAN, N., NISSEN, P., HANSEN, J., MOORE, P. B. & STEITZ, T. A. 2000. The complete atomic structure of the large ribosomal subunit at 2.4 Å resolution. *Science,* 289**,** 905-920.

BARKER, A. F. 2002. Bronchiectasis. *New England Journal of Medicine,* 346**,** 1383-1393.

BARNES, P. J. 2007. Chronic obstructive pulmonary disease: A growing but neglected global epidemic. *PLoS Medicine,* 4**,** 779-780.

BARNES, P. J., SHAPIRO, S. D. & PAUWELS, R. A. 2003. Chronic obstructive pulmonary disease: molecular and cellular mechanisms. *European Respiratory Journal,* 22**,** 672-688.

BAUS, S. & FENTON, M. J. 2004. Toll-like receptors: Function and roles in lung disease. *American Journal of Physiology: Lung Cellular and Molecular Physiology,* 286**,** 887-892.

BEARD, W. A., SHOCK, D. D., VANDE BERG, B. J. & WILSON, S. H. 2002. Efficiency of correct nucleotide insertion governs DNA polymerase fidelity. *The Journal of Biological Chemistry,* 277**,** 47393-47398.

BEARD, W. A. & WILSON, S. H. 2000. Structural design of a eukaryotic DNA repair polymerase: DNA polymerase β. *Mutations Research,* 460**,** 231-244.

BEARD, W. A. & WILSON, S. H. 2003. Structural insights into the origins of DNA polymerase fidelity. *Structure,* 11**,** 489-496.

BEASLEY, V., JOSHI, P. V., SINGANAYAGAM, A., MOLYNEAUX, P. L., JOHNSTON, S. L. & MALLIA, P. 2012. Lung microbiology and exacerbations in COPD. *International Journal of COPD,* 7**,** 555-569.

BEBENEK, K. & KUNKEL, T. A. 2004. Functions of DNA polymerases. *Advances in Protein Chemistry,* 69**,** 137-163.

BEESE, L. S., DERBYSHIRE, V. & STEITZ, T. A. 1993. Structure of DNA polymerase I Klenow fragment bound to duplex DNA. *Science,* 260**,** 352-355.

BERESFORD, T. & CONDON, S. 1991. Cloning and partial characterisation of genes for ribonucleic acid in *Lactococcus lactis* subsp. *lactis*. *FEMS Microbiology Letters,* 62**,** 319-323.

BERNASCONI, E., VALSANGIACOMO, C., PEDUZZI, R., CAROTA, A., MOCCETTI, T. & FUNKE, G. 2004. *Arthrobacter woluwensis* subacute infective endocarditis: Case report and review of the literature. *Clinical Infectious Diseases,* 38**,** 27-31.

BERNEY, C., FAHRNI, J. & PAWLOWSKI, J. 2004. How many novel eukaryotic 'kingdoms'? Pitfalls and limitations of environmental DNA surveys. *BMC Biology,* 2**,** 13.

BITTAR, F., RICHET, H., DUBUS, J. C., REYNAUD-GAUBERT, M., STREMLER, N., SARLES, J., RAOULT, D. & ROLAIN, J. M. 2008. Molecular detection of multiple emerging pathogens in sputa from cystic fibrosis patients. *PLoS ONE,* 3**,** e2908.

BODAGHI, B., DAUGA, C., CASSOUX, N., WECHSLER, B., MERLE-BERAL, H., POVEDA, J. D., PIETTE, J. C. & LEHOANG, P. 1998. Whipple's syndrome (uveitis, B27-negative spondylarthropathy, meningitis, and lymphadenopathy) associated with *Arthrobacter* sp. infection. *Ophthalmology,* 105**,** 1891-1896.

BOGAERT, D., VAN BELKUM, A., SLUIJTER, M., LUIJENDIJK, A., DE GROOT, R., RÜMKE, H. C., VERBRUGH, H. A. & HERMANS, P. W. M. 2004. Colonisation by *Streptococcus pneumoniae* and *Staphylococcus aureus* in healthy children. *Lancet,* 363**,** 1871-1872.

BONTON, M. J. M., VAN TIEL, F. H., VAN DER GEEST, S., SMEETS, H. G. W., STOBBERINGH, E. E. & GAILLARD, C. A. 1993. Topical antimicrobial prophylaxis of nosocomial pneumonia in mechanically ventilated patients. Microbiological observations. *Infection,* 21**,** 137-139.

BOUCHARA, J. P., HSIEH, H. Y., CROQUEFER, S., BARTON, R., MARCHAIS, V., PIHET, M. & CHANG, T. C. 2009. Development of an oligonucleotide array for direct detection of fungi in sputum samples from patients with cystic fibrosis. *Journal of Clinical Microbiology,* 47.

BOUCHER, R. C. 2002. An overview of the pathogenesis of cystic fibrosis lung disease. *Advanced Drug Delivery Reviews,* 54**,** 1359-1371.

BOURKE, S. J. 2002. Respiratory Tract Infections: A Clinical Overview. *In:* SUSSMAN, M. (ed.) *Molecular Medical Microbiology.* London: Academic Press.

BOWIE, A. & O'NEIL, L. A. 2000. The interleukin-1 receptor/Toll-like receptor superfamily: Signal generators for pro-inflammatory interleukin and microbial products. *Journal of Leukocyte Biology,* 67**,** 508-514.

BRADLEY, R. D. & HILLIS, D. M. 1997. Recombinant DNA sequences generated by PCR amplification. *Molecular Biology and Evolution,* 14.

BRAKSTAD, O. G., NONSTAD, I., FAKSNESS, L. G. & BRANDVIK, P. J. 2008. Responses of microbial communities in artic sea ice after contamination by crude petroleum oil. *Microbial Ecology,* 55**,** 540-552.

BRAUTIGAM, C. A. & STEITZ, T. A. 1998. Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. *Current Opinion in Structural Biology,* 8**,** 54-63.

BRINKHOFF, T. & VAN HANNEN, E. J. 2001. Use of silicone grease to avoid "smiling effect" in denaturing gradient gel electrophoresis. *Journal of Rapid Methods and Automation in Microbiology,* 9**,** 259-261.

BRITISH LUNG FOUNDATION 2006. Lost in translation: Bridging the communication gap in COPD.

BROGDEN, K. A., GUTHMILLER, J. M. & TAYLOR, C. E. 2005. Human polymicrobial infections. *Lancet,* 365**,** 253-255.

BROWN, C. J., WONG, M., DAVIS, C. C., KANTI, A., ZHOU, X. & FORNEY, L. J. 2007. Preliminary chacterisation of the normal microbiota of the human vulva using cultivation-independent methods. *Journal of Medical Microbiology,* 56**,** 271-276.

BRUMFIT, W., WILLOUGHBY, M. & BROMLEY, L. 1957. Evaluation of sputum in chronic bronchitis. *Lancet,* 270**,** 1306-1309.

BURROWS, B. & EARLE, R. H. 1969. Course and prognosis of chronic obstructive lung disease: A prospective study of 200 patients. *The New England Journal of Medicine,* 280**,** 397-404.

CABELLO, H., TORRES, A., CELIS, R., EL-EBIARY, M., DE LA BELLACASA, J. P., XAUBET, J., GONZALEZ, J., AGUSTÍ, C. & SOLER, N. 1997. Bacterial colonisation of distal airways in healthy subjects and chronic lung disease: A bronchoscopic study. *European Respiratory Journal,* 10**,** 1137-1144.

CABRERA-RUBIO, R., GARCIA-NÙÑEZ, M., SETÓ, L., ANTÓ, J. M., MOYA, A., MONSÓ, E. & MIRA, A. 2012. Microbiome diversity in the bronchial tracts of patients with chronic obstructive pulmonary disease. *Journal of Clinical Microbiology,* 50**,** 3562-3568.

CATE, J. H., YUSUPOV, M. M., YUSUPOVA, G. Z., EARNEST, T. N. & NOLLER, H. F. 1999. X-ray crystal structures of 70*S* ribosome functional complexes. *Science,* 285**,** 2095-2104.

CHAKRAVORTY, S., HELB, D., BURDAY, M., CONNELL, N. & ALLAND, D. 2007. A detailed analysis of 16S ribosomal RNA gene segments for the diagnosis of pathogenic bacteria. *Journal of Microbiological Methods,* 69**,** 330-339.

CHAN, P. K., TAM, J. S., LAM, C. W., CHAN, E., WU, A., LI, C. K., BUCKLEY, T. A., NG, K. C., JOYNT, G. M., CHENG, F. W. T. & OTHERS 2003. Human metapneumovirus detection in patients with severe acute respiratory syndrome. *Emerging Infectious Diseases,* 9**,** 1058-1063.

CHARLSON, E. S., CHEN, J., CUSTERS-ALLEN, R., BITTINGER, K., LI, H., SINHA, R., HWANG, J., BUSHMAN, F. D. & COLLMAN, R. G. 2010. Disordered microbial communities in the upper respiratory tract of cigarette smokers. *PLos One,* 5**,** e15216.

CHEN, F. L., WANG, G. C., TENG, S. O., OU, T. Y., YU, F. L. & LEE, W. S. 2012. Clinical and epidemiological features of *Chryseobacterium indologenes* infections: Analysis of 215 cases (in press). *Journal of Microbiology, Immunology and Infection* [Online].

CHIEN, A., EDGAR, D. B. & TRELA, J. M. 1976. Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *Journal of Bacteriology,* 127**,** 1550-1557.

CHOTIRMALL, S. H., O'DONOGHUE, E., BENNETT, K., GUNARATNAM, C., O'NEIL, S. J. & MCELVANEY, N. G. 2010. Sputum *Candida albicans* presages FEV_1 decline and hospital-treated exacerbations in cystic fibrosis. *Chest,* 138**,** 1186-1195.

CHRISTNER, B. C., MOSLEY-THOMPSON, E., THOMPSON, L. G. & REEVE, J. N. 2001. Isolation of bacteria and 16*S* rDNAs from Lake Vostok accretion ice. *Environmental Microbiology,* 3**,** 570-577.

CIMON, B., CARRERE, J., VINATIER, J. F., CHAZALETTE, J. P., CHABASSE, D. & BOUCHARA, J. P. 2000. Clinical significance of *Scedosporium apiospermum* in patients with cystic fibrosis. *European Journal of Clinical Microbiology & Infectious Diseases,* 19**,** 53-56.

CLINE, J., BRAMAN, J. C. & HOGREFE, H. H. 1996. PCR fidelity of *Pfu* DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Research,* 24**,** 3546-3551.

COHEN, M. & SAHN, S. A. 1999. Bronchiectasis in systemic diseases. *Chest,* 116**,** 1063-1074.

COLE, P. J. 1986. Inflammation: a two-edged sword--the model of bronchiectasis. *European Journal of Respiratory Diseases. Supplement,* 147**,** 6-15.

COREY, M., EDWARDS, L., LEVISON, H. & KNOWLES, M. 1997. Longitudinal analysis of pulmonary function in patients with cystic fibrosis. *The Journal of Pediatrics,* 131**,** 809-814.

COSTELLO, E. K., LAUBER, C. L., HAMADY, M., FIERER, N., GORDON, J. I. & KNIGHT, R. 2009. Bacterial community variation in human body habitats across space and time. *Science,* 326**,** 1694-1697.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. P. 1999. Bacterial biofilms: A common cause of persistant infections. *Science,* 284**,** 1318-1322.

COTES, J. E., CHINN, D. J. & MILLER, M. R. 2006a. Genetic diversity: Reference values in non-Caucasians. *Lung Function: Physiology, Measurement and Application in Medicine.* Blackwell Publishing Ltd.

COTES, J. E., CHINN, D. J. & MILLER, M. R. 2006b. Reference values for lung function in white (Caucasian) children and adults. *Lung Function: Physiology, Measurement and Application in Medicine.* 6th ed.: Blackwell Publishing Ltd.

COX, M. J., ALLGAIER, M., TAYLOR, B., BAEK, M. S., HUANG, Y. J., DALY, R. A., KARAOZ, U., ANDERSEN, G. L., BROWN, R., FUJIMURA, K. E. & ET AL 2010. Airway microbiota and pathogen abundance in age-stratified cystic fibrosis patients. *PloS One,* 5**,** e11044.

CURTIS, T. P., SLOAN, W. T. & SCANNELL, J. W. 2002. Estimating prokaryotic diversity and its limits. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 10494-10499.

CYSTIC FIBROSIS FOUNDATION. *About Cystic Fibrosis* [Online]. Available: http://www.cff.org/AboutCF/ [Accessed 17th March 2012].

DAHLLÖF, I., BAILLIE, H. & KJELLEBERG, S. 2000. *rpoB*-based microbial community analysis avoids limitations inherent in 16S rRNA gene intraspecies heterogeneity. *Applied and Environmental Microbiology,* 66**,** 3376-3380.

DAVIES, J. C. & BILTON, D. 2009. Bugs, biofilms, and resistance in cystic fibrosis. *Respiratory Care,* 54**,** 628-640.

DAVIS, G., WELLS, A. U., DOFFMAN, S., WATANABE, S. & WILSON, R. 2006. The effect of *Pseudomonas aeruginosa* on pulmonary function in patients with bronchiectasis. *European Respiratory Journal,* 28**,** 974-979.

DE BENTZMANN, S., ROGER, P. & PURCHELLE, E. 1996. *Pseudomonas aeruginosa* adherence to remodelling respiratory epithelium. *European Respiratory Journal,* 9**,** 2145-2150.

DE VRANKRIJKER, A. M. M., WOLFS, T. F. W. & VAN DER ENT, C. K. 2010. Challenging and emerging pathogens in cystic fibrosis. *Paediatric Respiratory Reviews,* 11**,** 246-254.

DECRAMER, M., RENNARD, S., TROOSTERS, T., MAPEL, D. W., GIARDINO, N., MANNINO, D., WOUTERS, E., SETHI, S. & COOPER, G. B. 2008. COPD as a lung disease with systemic consequences: clinical impact, mechanisms, and potential for early intervention. *Journal of Chronic Obstructive Pulmonary Disease,* 5**,** 235-256.

DELARUE, M., POCH, O., TORDO, N., MORAS, D. & ARGOS, P. 1990. An attempt to unify the structure of polymerases. *Protein Engineering,* 3**,** 461-467.

DELONG, E. F. & PACE, N. R. 2001. Environmental diversity of bacteria and archaea. *Systematic Biology,* 50**,** 470-478.

DELONG, E. F., WU, K. Y., PREZELIN, B. B. & JOVINE, R. V. 1994. High abundance of Archaea in Antarctic marine picoplankton. *Nature,* 371**,** 695-697.

DETHLEFSEN, L., HUSE, S., SOGIN, M. L. & RELMAN, D. A. 2008. The pervasive effects of an anitbiotic on the human gut microbiota, as revealed by deep 16S rRNA sequencing. *PLoS Biology,* 6**,** e280.

DETHLEFSEN, L., MCFALL-NGAI, M. & RELMAN, D. A. 2007. An ecological and evolutionary perspective on human-microbe mutualism and disease. *Nature,* 449**,** 811-818.

DIAZ, P. I., CHALMERS, N. I., RICKARD, A. H., KONG, C., MILBURN, C. L., PALMER, R. J., JR. & KOLENBRANDER, P. E. 2006. Molecular characterisation of subject-specific oral microflora during initial colonisation of enamel. *Applied and Environmental Microbiology,* 72**,** 2837-2848.

DOLLHOPF, S. L., HASHSHAM, S. A. & TIEDJE, J. M. 2001. Interpreting 16S rDNA T-RFLP data: Application of self-organising maps and principal component analysis to describe community dynamics and convergence. *Microbial Ecology,* 42**,** 495-505.

DON, R. H., COX, P. T., WAINWIRGHT, B. J., BAKER, K. & MATTICK, J. S. 1991. "Touchdown" PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Research,* 19**,** 4008.

DONLAN, R. M. & COSTERTON, J. W. 2002. Biofilms: Survival mechanisms of clinically relevant micro-organisms. *Clinical Microbiology Reviews,* 15**,** 167-193.

DÖRING, G. & RATJEN, F. 2005. Cystic fibrosis molecular cell biology. *In:* MEYERS, R. A. (ed.) *Encylopedia of Molecular Cell Biology and Molecular Medicine.* 2nd ed. Weinheim: Wiley-VCH.

DOWD, S. E., CALLAWAY, T. R., WOLCOTT, R. D., SUN, Y., MCKEEHAN, T., HAGEVOORT, R. G. & EDRINGTON, T. S. 2008. Evaluation of the bacterial diversity in the faeces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyosequencing (bTEFAP). *BMC Microbiology,* 8**,** 125.

DRENNAN, M. B., NICOLLE, D., QUESNIAUX, V. J. F., JACOBS, M., ALLIE, N., MPAGI, J., FRÉMOND, C., WAGNER, H., KIRSCHNING, C. & RYFFEL, B. 2004. Toll-like receptor 2 deficient mice succumb to *Mycobacterium tuberculosis* infection. *American Journal of Pathology,* 164**,** 49-57.

DUMBRELL, A. J., NELSON, M., HELGASON, T., DYTHAM, C. & FITTER, A. H. 2010. Relative roles of niche and neutral processes in structuring a soil microbial community. *ISME,* 4**,** 337-45.

DYKHUIZEN, D. E. 1998. Santa Rosalia revisted: Why are they so many species of bacteria? *Antonie Van Leeuwenhoek,* 73**,** 25-33.

ECKBURG, P. B., BIK, E. M., BERNSTEIN, C. N., PURDOM, E., DETHLEFSEN, L., SARGENT, M., GIL, S. R., NELSON, K. E. & RELMAN, D. A. 2005. Diversity of the human intestinal microbial flora. *Science,* 308**,** 1635-1638.

ECKBURG, P. B. & RELMAN, D. A. 2007. The role of microbes in Crohn's disease. *Clinical Infectious Diseases,* 44**,** 256-262.

EDENBORN, S. L. & SEXSTONE, A. J. 2007. DGGE fingerprinting of culturable soil bacterial communities complements culture-independent analyses. *Soil Biology & Biochemistry,* 39**,** 1570-1579.

EDWARDS, R., RODRIGUEZ-BRITO, B., WEGLEY, L., HAYNES, M., BREITBART, M., PETERSON, D., SAAR, M., ALEXANDER, S., ALEXANDER, E. & ROHWER, F. 2006. Using pyrosequencing to shed light on deep mine microbial ecology. *BMC Genomics,* 7**,** 57.

EILERS, H., PERNTHALER, J., GLÖCKNER, F. O. & AMANN, R. 2000. Culturability and *in situ* abundance of pelagic bacteria from the North Sea. *Applied and Environmental Microbiology,* 66**,** 3044-3051.

ERB-DOWNWARD, J. R., THOMPSON, D. L., HAN, M. K., FREEMAN, C. M., MCCLOSKEY, L., SCHMIDT, L. A., YOUNG, V. B., TOEWS, G. B., CURTIS, J. L., SUNDARAM, B., MARTINEZ, F. J. & HUFFNAGLE, G. B. 2011. Analysis of the lung microbiome in the "healthy" smoker and in COPD. *PLos One,* 6**,** e16384.

ESTEBAN, J., BUENO, J., PEREZ-SANTONJA, J. J. & SORIANO, F. 1996. Endophthalmitis involving an *Arthrobacter*-like organism following intraocular lens implantation. *Clinical Infectious Diseases,* 23**,** 1180-1181.

FAGON, J. Y., CHASTRE, J., TROUILLET, J. L., DOMART, Y., DOMBRET, M. C., BORNET, M. & GIBERT, C. 1990. Characterisation of distal microflora during acute exacerbations of chronic bronchitis. *American Review of Respiratory Disease,* 142**,** 1004-1008.

FEGAN, M., FRANCIS, P., HAYWARD, A. C., DAVIS, G. H. & FUERST, J. A. 1990. Phenotypic conversion of *Pseudomonas aeruginosa* in cystic fibrosis. *Journal of Clinical Microbiology,* 28**,** 1143-1146.

FERRIS, M. J. & WARD, D. M. 1997. Seasonal distributions of dominant 16S rRNA-defined populations in a hot spring microbial mat examined by denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology,* 63**,** 1375-1381.

FIERER, N., HAMADY, M., LAUBER, C. L. & KNIGHT, R. 2008. The influence of sex, handedness, and washing on the diversity of hand surface bacteria. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 17994-17999.

FINLEY, T. N. & LADMAN, A. J. 1972. Low yield of pulmonary surfactant in cigarette smokers. *The New England Journal of Medicine,* 286**,** 223-227.

FISCHER, S. G. & LERMAN, L. S. 1983. DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: Correspondence with melting theory. *Proceedings of the National Academy of Sciences of the United States of America,* 80**,** 1579-1583.

FOWERAKER, J. E. 2009. Recent advances in the microbiology of respiratory tract infection in cystic fibrosis. *British Medical Bulletin,* 89**,** 93-110.

FOWERAKER, J. E., LAUGHTON, C. R., BROWN, D. F. J. & BILTON, D. 2005. Phenotypic variability of *Pseudomonas aeruginosa* in sputa from patients with acute infective exacerbation of cystic fibrosis and its impact on the validity of antimicrobial susceptibility testing. *Journal of Antimicrobial Chemotherapy,* 55**,** 921-927.

FOXMAN, B., GOLDBERG, D., MURDOCK, C., XI, C. & GILSDORF, J. R. 2008. Conceptualising human microbiota: From multicelled organ to ecological community. *Interdisciplinary Perspectives on Infectious Diseases,* 2008**,** 1-5.

FUJIMOTO, C., MAEDA, H., KOKEGUCHI, S., TAKASHIBA, S., NISHIMURA, F., ARAI, H., FUKUI, K. & MURAYAMA, Y. 2003. Application of denaturing gradient gel electrophoresis (DGGE) to the analysis of microbial communities of subgingival plaque. *Journal of Peridontal Research,* 38**,** 440-445.

FUNKE, G., HUTSON, R. A., BERNARD, K. A., PFYFFER, G. E., WAUTER, G. & COLLINS, M. D. 1996. Isolation of *Arthrobacter* spp. from clinical specimens and description of *Arthrobacter cumminsii* sp. nov. and *Arthrobacter woluwensis* sp. nov. *Journal of Clinical Microbiology,* 34**,** 2356-2363.

FUTTERER, O., ANGELOV, A., LIESEGANG, H., GOTTSCHALK, G., SCHLEPER, C., SCHEPERS, B., DOCK, C., ANTRANIKAN, G. & LIEBL, W. 2004. Genome sequence of *Picrophilus torridus* and its implications for life around pH 0. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 9091-9096.

GAO, Z., TSENG, C. H., PEI, Z. & BLASER, M. J. 2007. Molecular analysis of the human forearm superficial skin bacterial biota. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 2927-2932.

GARAU, J. & GOMEZ, L. 2003. *Pseuodmonas aeruginosa* pneumonia. *Current Opinion in Infectious Diseases,* 16**,** 135-143.

GARNIER, T., CANARD, B. & COLE, S. T. 1991. Cloning, mapping, and molecular characterisation of the rRNA operon of *Clostridium perfingens*. *Journal of Bacteriology,* 173**,** 5431-5438.

GEORGE, A. M., JONES, P. M. & MIDDLETON, P. G. 2009. Cystic fibrosis infections: Treatment strategies and prospects. *FEMS Microbiology Letters,* 300**,** 153-164.

GHANNOUM, M. A., JUREVIC, R. J., MUKHERJEE, P. K., CUI, F., SIKAROODI, M., NAQVI, A. & GILLEVET, P. M. 2010. Characterisation of the oral fungal microbiome (mycobiome) in healthy individuals. *PLoS Pathogens,* 6**,** e1000713.

GOERING, R. V., DOCKRELL, H. M., ZUCKERMAN, M., WAKELIN, D., ROITT, I. M., MIMS, C. & CHIODINI, P. L. 2008. *Mims' Medical Microbiology,* China, Mosby, Elsevier Ltd.

GOLDENBERG, S. & PRICE, N. 2008. Opportunistic fungal lung infections. *Medicine,* 36**,** 295-299.

GONZALEZ, A., CLEMENTE, J. C., SHADE, A., METCALF, J. L., SONG, S., PRITHIVIRAJ, B., PALMER, B. E. & KNIGHT, R. 2011. Our microbial selves: What ecology can teach us. *EMBO Reports,* 12**,** 775-784.

GORDON, D. A., PRISCU, J. & GIOVANNONI, S. J. 2000. Origin and phylogeny of microbes living in permanent Antarctic lake ice. *Microbial Ecology,* 39**,** 197-202.

GREENSILL, J., MCNAMARA, P. S., DOVE, W., FLANAGAN, B., SMYTH, R. L. & HART, C. A. 2003. Human metapneumovirus in severe respiratory syncytial virus bronchiolitis. *Emerging Infectious Diseases,* 9**,** 372-375.

GRIMWOOD, K. 2011. Airway microbiology and host defences in paediatric non-CF bronchiectasis. *Paediatric Respiratory Reviews,* 12**,** 111-118.

GROENEVELD, K., VAN ALPEN, L., EIJK, P. P., JANSEN, H. M. & ZANEN, H. C. 1988. Changes in outer membrane proteins of non-typeable *Haemophilus influenzae* in patients with chronic obstructive pulmonary disease. *Journal of Infectious Diseases,* 158**,** 360-365.

GUMP, D. W., PHILLIPS, C. A., FORSYTH, B. R., MCINTOSH, K., LAMBORN, K. R. & STOUCH, W. H. 1976. Role of infection in chronic bronchitis. *American Review of Respiratory Disease,* 113**,** 465-474.

HAASE, G., SKOPNIK, H. & KUSENBACH, G. 1990. *Exophiala dermatitidis* infection in cystic fibrosis. *Lancet,* 336**,** 188-189.

HALL-STOODLEY, L., COSTERTON, J. W. & STOODLEY, P. 2004. Bacterial biofilms: From the environment to infectious disease. *Nature Reviews Microbiology,* 2**,** 95-108.

HALL-STOODLEY, L. & STOODLEY, P. 2005. Biofilm formation and dispersal and the trasnmission of human pathogens. *Trends in Microbiology,* 1**,** 7-10.

HALL-STOODLEY, L. & STOODLEY, P. 2009. Evolving concepts in biofilm infections. *Cellular Microbiology,* 11**,** 1034-1043.

HAMADY, M. & KNIGHT, R. 2009. Microbial community profiling for human microbiome projects: Tools, techniques, and challenges. *Genome Research,* 19**,** 1141-1152.

HAMMER, Ø. & HARPER, D. A. T. 2001. PAST: Palaeontological Statistics software package for education and data analysis. *Palaeontologia Electronica,* 4**,** 9.

HAN, M. K., HUANG, Y. J., LIPUMA, J. J., BOUSHEY, H. A., BOUCHER, R. C., COOKSON, W. O., CURTIS, J. L., ERB-DOWNWARD, J. R., LYNCH, S. V.,

SETHI, S., TOWES, G. B., YOUNG, V. B., WOLFGANG, M. C., HUFFNAGLE, G. B. & MARTINEZ, F. J. 2012. Significance of the microbiome in obstructive lung disease. *Thorax,* 67**,** 456-463.

HARRIS, J. K., DE GROTTE, M. A., SAGEL, S. D., ZEMANICK, E. T., KAPSNER, R., PENVARI, C., KAESS, H., DETERDING, R. R., ACCURSO, F. J. & PACE, N. R. 2007. Molecular identification of bacteria in bronchoalveolar lavage fluid from children with cystic fibrosis. *Proceedings of the National Academy of Sciences of the United States of America,* 104**,** 20529-20533.

HASSETT, D. J., CUPPOLETTI, J., TRAPNELL, B., LYMAR, S. V., ROWE, J. J., YOON, S. S., HILLIARD, G. M., PARVATIYAR, K., KAMANI, M. C., WOZNIAK, D. J., HWANG, S. H., MCDERMOTT, T. R. & OCHSNER, U. A. 2002. Anaerobic metabolism and quorum sensing by *Pseudomonas aeruginosa* biofims in chronically infected cystic fibrosis airways: Rethinking anitbiotic treatment strategies and drug targets. *Advanced Drug Delivery Reviews,* 54**,** 1425-1443.

HEDEGAARD, J., OKKELS, H., BRUUN, B., KILIAN, M., MORTENSEN, K. K. & NØRSKOV-LAURITSEN, N. 2001. Phylogeny of the genus *Haemophilus* as determined by comparison of partial *infB* sequences. *Microbiology,* 147**,** 2599-2609.

HILL, G. B. 1993. The microbiology of bacterial vaginosis. *American Journal of Obstetrics and Gynecology,* 169**,** 450-454.

HILTY, M., BURKE, C., PEDRO, H., CARDENAS, P., BUSH, A., BOSSLEY, C., DAVIES, J., ERVINE, A., POULTER, L., PACHTER, L., MOFFATT, M. F. & COOKSON, W. O. C. 2010. Disordered microbial communities in asthmatic airways. *PLoS ONE,* 5**,** e8578.

HINDRÉ, T., KNIBBE, C., BELSON, G. & SCHNEIDER, D. 2012. New insights into bacterial adaptation through *in vivo* and *in silico* experimental evolution. *Nature Reviews Microbiology,* 10**,** 352-365.

HOCHREIN, H., SCHLATTER, B., O'KEEFFE, M., WAGNER, C., SCHMITZ, F., SCHIEMANN, M., BAUER, S., SUTER, M. & WAGNER, H. 2004. Herpes simplex virus type-1 induces IFN-α production via Toll-like receptor 9-dependent and -independent pathways. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 11416-11421.

HOLST, D. 2005. Causes and prevention of dental caries: a perspective on cases and incidence. *Oral Health and Preventative Dentistry,* 3**,** 9-14.

HONDA, Y., TAKAHASHI, H., KUROKI, Y., AKINO, T. & ABE, S. 1996. Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. *Chest,* 109**,** 1006-1009.

HORZ, H. P., ROTTHAUWE, J. H., LUKOW, T. & LIESACK, W. 2000. Identification of major subgroups of ammonia-oxidising bacteria in environmental samples by T-RFLP analysis of *amoA* PCR products. *Journal of Microbiological Methods,* 39**,** 197-204.

HOVIG, E., SMITH-SØRENSEN, B., BRØGGER, A. & BØRRESEN, A. L. 1991. Constant denaturant gel-electrophoresis, a modification of denaturing gradient gel-electrophoresis, in mutation detection. *Mutations Research Letters,* 262**,** 63-71.

HSU, C. L., SHIH, L. Y., LEU, H. S., WU, C. L. & FUNKE, G. 1998. Septicaemia due to *Arthrobacter* species in a neutropaenic patient with acute lymphoblastic leukaemia. *Clinical Infectious Diseases,* 27**,** 1334-1335.

HSUEH, P. R., HSIUE, T. R., WU, J. J., TENG, L. J., HO, S. W., HSIEH, W. C. & LUH, K. T. 1996. *Flavobacterium indologenes* bacteraemia: Clinical and microbiological characteristics. *Clinical Infectious Diseases,* 23**,** 550-555.

HUANG, Y. J., KIM, E., COX, M. J., BRODIE, E. L., BROWN, R., WIENER-KRONISH, J. P. & LYNCH, S. V. 2010. A persistent and diverse airway microbiota present during chronic obstructive pulmonary disease exacerbations. *OMICS A Journal of Integrative Biology,* 14**,** 9-59.

HUANG, Y. J. & LYNCH, S. V. 2011. The emerging relationship between the airway microbiota and chronic respiratory disease: Clinical implications. *Expert Reviews of Respiratory Medicine,* 6**,** 809-821.

HUBER, R., HUBER, H. & STETTER, K. O. 2000. Towards the ecology of hyperthermophiles: Biotopes, new isolation strategies and novel metabolic properties. *FEMS Microbiology Reviews,* 24**,** 615-623.

HUGENHOLTZ, P., GOEBEL, B. M. & PACE, N. R. 1998a. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. *Journal of Bacteriology,* 180**,** 4765-4774.

HUGENHOLTZ, P. & HUBER, T. 2003. Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *International Journal of Systematic and Evolutionary Microbiology,* 53**,** 289-293.

HUGENHOLTZ, P., PITULLE, C., HERSCHBERGER, K. L. & PACE, N. R. 1998b. Novel division level bacteria diversity in a Yellowstone hot spring. *Journal of Bacteriology,* 180**,** 366-376.

HUGHES, J. B., HELLMANN, J. J., RICKETTS, T. H. & BOHANNAN, B. J. M. 2001. Counting the uncountable: Statistical approaches to estimating microbial diversity. *Applied and Environmental Microbiology,* 67**,** 4399-4406.

HUGHES, J. M. B. 2007. Pulmonary function: The basics. *Medicine,* 36**,** 142-150.

JACKSON, C. R., RODEN, E. E. & CHURCHILL, P. F. 2000. Denaturing gradient gel electrophoresis can fail to separate 16S rDNA fragments with multiple base differences. *Molecular Biology Today,* 1**,** 49-51.

JANSE, I., BOK, J. & ZWART, G. 2004. A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *Journal of Microbiological Methods,* 57**,** 279-281.

JARJOUR, N. N. & KELLY, E. A. B. 2002. Pathogenesis of asthma. *The Medical Clinics of North America,* 86**,** 925-936.

JONASSON, J., OLOFSSON, M. & MONSTEIN, H. J. 2002. Classification, identification and subtyping of bacteria based on pyrosequencing and signature matching of 16S rDNA fragments. *APMIS,* 110**,** 263-72.

JOYCE, C. M. & STEITZ, T. A. 1994. Function and structure relationships in DNA polmerases. *Annual Review of Biochemistry,* 63**,** 777-822.

JUDO, M. S. B., WEDEL, A. B. & WILSON, C. 1998. Stimulation and suppression of PCR-mediated recombination. *Nucleic Acids Research,* 26**,** 1819-1825.

KACZANOWSKA, M. & RYDÉN-AULIN, M. 2007. Ribsome biogenesis and the translation process in *Escherichia coli*. *Microbiology and Molecular Reviews,* 71**,** 477-494.

KALEDIN, A. S., SLIUSARENKO, A. G. & GORODETSKII, S. I. 1980. Isolation and properties of DNA polymerase from extreme thermophilic bacteria *Thermus aquaticus* YT-1s. *Biokhimiia,* 45**,** 644-651.

KELLER, M. & ZENGLER, K. 2004. Tapping into microbial diversity. *Nature Reviews Microbiology,* 2**,** 141-150.

KELLER, N. P. & HOHN, T. M. 1997. Metabolic pathway gene clusters in filamentous fungi. *Fungal Gentics and Biology,* 21**,** 17-29.

KEREM, B. S., ROMMENS, J. M., BUCHANAN, J. A., MARKIEWICZ, D., COX, T. K., CHAKRAVARTI, A., BUCHWALD, M. & TSUI, L. C. 1989. Identification of the cystic fibrosis gene: Genetic analysis. *Science,* 245**,** 1073-1080.

KEREM, E., REISMAN, J., COREY, M., CANNY, G. J. & LEVISON, H. 1992. Prediction of mortality in patients with cystic fibrosis. *The New England Journal of Medicine,* 326**,** 1187-1191.

KIEFER, J. R., MAO, C., BRAMAN, J. C. & BEESE, L. S. 1998. Visualsing DNA replication in a catalytically active *Bacillus* DNA polymerase crystal. *Nature,* 391**,** 304-307.

KING, P. T. 2011a. Pathogenesis of bronchiectasis. *Paediatric Respiratory Reviews,* 12**,** 104-110.

KING, P. T. 2011b. The pathophysiology of bronchiectasis. *International Journal of COPD,* 4**,** 411-419.

KING, P. T., HOLDSWORTH, S. R., FREEZER, N. J., VILLANUEVA, E., GALLAGHER, M. & HOLMES, P. W. 2005. Outcome in adult bronchiectasis. *COPD: Journal of Chronic Obstructive Pulmonary Disease,* 2**,** 27-34.

KING, P. T., HOLDSWORTH, S. R., FREEZER, N. J., VILLANUEVA, E. & HOLMES, P. W. 2006. Characterisation of the onset and presenting clinical features of adult bronchiectasis. *Respiratory Medicine,* 100**,** 2183-2189.

KING, P. T., HOLDSWORTH, S. R., FREEZER, N. J., VILLANUEVA, E. & HOLMES, P. W. 2007. Microbiologic follow-up study in adult bronchiectasis. *Respiratory Medicine,* 101**,** 1633-1638.

KINKE, T., KNEIST, S., DE SOET, J. J., KUHLISCH, E., MAUERBERGER, S., FÖSTER, A. & KLIMM, W. 2009. Acid production by oral strains of *Candida albicans* and lactobacilli. *Caries Research,* 43**,** 83-91.

KISS, A., SAIN, B. & VENETIANER, P. 1977. The number of rRNA genes in *Escherichia coli*. *FEBS Letters,* 79**,** 77-79.

KLEPAC-CERAJ, V., LEMON, K. P., MARTIN, T. R., ALLGAIER, M., LORY, S., BRODIE, E. L., LYNCH, S. V., BOHANNAN, B. J. M., GREEN, J. L., MAURER, B. A. & KOLTER, R. 2010. Relationship between cystic fibrosis respiratory tract bacterial communities and age, genotype, antibiotics and *Pseudomonas aeruginosa*. *Environmental Microbiology,* 12**,** 1293-1303.

KLINGMAN, K. L., PYE, A., MURPHY, T. F. & HILL, S. L. 1995. Dynamics of respiratory tract colonisation by *Branhamella catarrhalis* in bronchiectasis. *American Journal of Respiratory and Critical Care Medicine,* 152**,** 1072-1078.

KNOOPKA, A. 2009. What is microbial community ecology? *ISME,* 3**,** 1223-1230.

KO, F. W. S., NG, T. K. C., LI, T. S. T., FOK, J. P. C., CHAN, M. C. H., WU, A. K. L. & HUI, D. S. C. 2005. Sputum bacteriology in patients with acute exacerbations of COPD in Hong Kong. *Respiratory Medicine,* 99**,** 454-460.

KOHLSTAEDT, L. A., WANG, J., FRIEDMAN, J. M., RICE, P. A. & STEITZ, T. A. 1992. Crystal structure at 3.5 Å of HIV-1 reverse transcriptase complexed with an inhibitor. *Science,* 256**,** 1781-1790.

KOLBERT, C. P. & PERSING, D. H. 1999. Ribosomal DNA sequencing as a tool for identification of bacterial pathogens. *Current Opinion in Microbiology,* 2**,** 299-305.

KREADER, C. A. 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. *Applied and Environmental Microbiology,* 62**,** 1102-1106.

KUNKEL, T. A. 2004. DNA replication fidelity. *The Journal of Biological Chemistry,* 279**,** 16895-16898.

LAËNNEC, R. T. H. 1962. *A treatise on the disase of the chest,* New York: Library of the New York Academy of Medicine, Hafner Publishing.

LAFUCI, G., WIDOM, R. L., EISNER, R. L., JARVIS, E. D. & RUDNER, R. 1986. Mapping of rRNA genes with integratable plasmids in *Bacillus subtilis*. *Journal of Bacteriology,* 165**,** 204-214.

LAMBERT, H. P. & STERN, H. 1972. Infective factors in exacerbations of bronchitis and asthma. *British Medical Journal,* 30**,** 323-327.

LANE, D. J., PACE, B., OLSEN, G. J., STAHL, D. A., SOGIN, M. L. & PACE, N. R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceedings of the National Academy of Sciences of the United States of America,* 82**,** 6955-6959.

LARSEN, M. V., JANNER, J. H., NIELSEN, S. D., FRIIS-MØLLER, A., RINGBAEK, T. & LANGE, P. 2009. Bacteriology in acute exacerbation of chronic obstructive pulmonary disease in patients admitted to hospital. *Scandinavian Journal of Infectious Diseases,* 41**,** 26-32.

LAWN, S. D. 2004. AIDS in Africa: The impact of co-infections on the pathogenesis of HIV-1 infection. *Journal of Infection,* 48**,** 1-12.

LAWYER, F. C., STOFFEL, S., SAIKI, R. K., MYAMBO, K., DRUMMOND, R. & GELFAND, D. H. 1989. Isolation, characterisation, and expression in *Escherichia coli* of the DNA polymerase gene from *Thermus aquaticus*. *The Journal of Biological Chemistry,* 264**,** 6427-6437.

LEACH, R. M. 2007. Symptoms and signs of respiratory disease. *Medicine,* 36**,** 119-125.

LEE, D. H., ZO, Y. G. & KIM, S. J. 1996. Non-radioactive method to study genetic profiles of natural bacterial communitites by PCR-single-strand-conformation polymorphism. *Applied and Environmental Microbiology,* 62**,** 3112-3120.

LEES, A. W. & MCNAUGHT, W. 1959. Bacteriology of the lower respiratory tract secretions: Sputum and upper respiratory tract secretions in "normals" and chronic bronchitis. *Lancet,* 274**,** 1112-1115.

LENTINO, J. R. & LUCKS, D. A. 1987. Nonvalue of sputum culture in the management of lower respiratory tract infections. *Journal of Clinical Microbiology,* 25**,** 758-762.

LI, Z., KOSOROK, M. R., FARRELL, P. M., LAXOVA, A., WEST, S. E. H., GREEN, C. G., COLLINS, J., ROCK, M. J. & SPLAINGARD, M. L. 2005. Longitudinal development of mucoid *Pseudomonas aeruginosa* infection and lung disease progression in children with cystic fibrosis. *The Journal of the American Medical Association,* 293**,** 581-588.

LOPEZ, A. D. & MURRAY, C. C. 1998. The global burden of disease, 1990-2020. *Nature Medicine,* 4**,** 1241-1243.

MADIGAN, M. T., MARTINKO, J. M., DUNLOP, P. V. & CLARK, D. P. 2009. *Brock Biology of Microorganisms,* London, Pearson Education Ltd.

MAI-PROCHNOW, A., LUCAS-ELIO, P., EGAN, S., THOMAS, T., WEBB, J. S., SANCHEZ-AMAT, A. & KJELLEBERG, S. 2008. Hydrogen peroxide linked to lysine oxidase activity facilitates biofilm differentiation and dispersal in several Gram-negative bacteria. *Journal of Bacteriology,* 190**,** 5493-5501.

MANSON, J. M., RAUCH, M. & GILMORE, M. S. 2008. The commensal microbiology of the gastrointestinal tract. *Advances in Experimental Medicine and Biology,* 635**,** 15-28.

MARGULIES, M., EGHOLM, M., ALTMAN, W. E., ATTIYA, S., BADER, J. S., BEMBEN, L. A., BERKA, J., BRAVERMAN, M. S., CHEN, Y. J., CHEN, Z. & ET AL 2005. Genome sequencing in microfabricated high-density picolitre reactors. *Nature,* 437**,** 376-380.

MARIEB, E. N. & HOEHN, K. 2007. *Human Anatomy & Physiology,* London, Pearson Education Ltd.

MARIN, A., MONSÓ, E., GARCIA-NÙÑEZ, M., SAULEDA, J., NOGUERA, A., PONS, J., AGUSTÍ, A. & MOREA, J. 2010. Variability and effects of bronchial colonisation in patients with moderate COPD. *European Respiratory Journal,* 35**,** 295-302.

MARTIN, D. W., SCHURR, M. J., MUDD, M. H. & DERETIC, V. 1993. Differentiation of *Pseudomonas aeruginosa* into the alginate-producing form: Inactivation of *mucB* causes conversion to mucoidy. *Molecular Microbiology,* 9**,** 497-506.

MARTÍNEZ-GARCÍA, M. A., SOLER-CATALUÑA, J. J., PERPIÑÁ-TORDERA, M., ROMÁN-SÁNCHEZ, P. & SORIANO, J. 2007. Factors associated with lung function decline in adult patients with stable non-cystic fibrosis bronchiectasis. *Chest,* 132**,** 1565-1572.

MAUGHAN, H., CUNNINGHAM, K. S., WANG, P. W., ZHANG, Y., CYPEL, M., CHAPARRO, C., TULLIS, D. E., WADELL, T. K., KESHAVJEE, S., LIU, M., GUTTMAN, D. S. & HWANG, D. M. 2012. Pulmonary bacterial communities in surgically re-sected non-cystic fibrosis bronchiectasis lungs are similar to those in cystic fibrosis. *Pulmonary Medicine,* Volume 2012**,** Article ID 746358.

MAYR, E. 1957. *The Species Problem,* Washington, D.C., American Association for the Advancement of Science.

MCHARDY, V. U., INGLIS, J. M., CALDER, M. A., CROFTON, J. W., GREGG, I., RYLAND, D. A., TAYLOR, P., CHADWICK, M., COOMBS, D. & RIDDELL, R. W. 1980. A study of infective and other factors in exacerbations of chronic bronchitis. *British Journal of Diseases of the Chest,* 74**,** 228-238.

MCPHERSON, J. D. 2009. Next-generation gap. *Nature Methods,* 6**,** S2-S5.

MEDZHITOV, R. & JANEWAY, C. A., JR. 1997. Innate immunity: Impact on the adaptive immune response. *Current Opinion in Immunology,* 9**,** 4-9.

MINAH, G. E. & LOESCHE, W. J. 1977. Sucrose metabolism by prominent members of the flora isolated from cariogenic and non-cariogenic dental plaques. *Infection and Immunity,* 17**,** 55-61.

MIRAVITLLES, M., ESPINOSA, C., FERNÁNDEZ-LASO, E., MARTOS, J. A., MALDONADO, J. A. & GALLEGO, M. 1999. Relationship between bacterial flora in sputum and functional impairment in patients with acute exacerbations of COPD. *Chest,* 116**,** 40-46.

MÖLLER, L. V. M., TIMENS, W., B.W., V. D., KOOI, K., DE WEVER, B., DANKERT, J. & VAN ALPHEN, L. 1998. *Haemophilus influenzae* in lung explants of patients with end-stage pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 157**,** 950-956.

MONSÓ, E., RUIZ, J., ROSELL, A., MANTEROLA, J., FIZ, J., MOREA, J. & AUSINA, V. 1995. Bacterial infection in chronic obstructive pulmonary disease: A study of stable and exacerbated outpatients using the protected specimen brush. *American Journal of Respiratory and Critical Care Medicine,* 152**,** 1316-1320.

MORRIS, A., SCIURBA, F. C., LEBEDEVA, I. P., GITHAIGA, A., ELLIOTT, W. M., HOGG, J. C., HUANG, L. & NORRIS, K. A. 2004. Association of chronic obstructive pulmonary disease severity and Pneumocystis colonisation. *American Journal of Respiratory and Critical Care Medicine,* 170**,** 408-413.

MORRISEY, B. M. 2007. Pathogenesis of bronchiectasis. *Clinics in Chest Medicine,* 28**,** 289-296.

MOSS, T., LANGLOSIS, F., GAGNON-KUGLER, T. & STEFANOVSKY, V. 2007. A housekeeper with power of attorney: The rRNA genes in ribosome biogenesis. *Cellular and Molecular Life Sciences,* 64**,** 29-49.

MURPHY, T. F., BRAUER, A. L., ESCHBERGER, K., LOBBINS, P., GROVE, L., CAI, X. & SETHI, S. 2008. *Pseudomonas aeruginosa* in chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 177**,** 853-860.

MURPHY, T. F., BRAUER, A. L., GRANT, B. J. B. & SETHI, S. 2005. *Moraxella catarrhalis* in chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 172**,** 195-199.

MURPHY, T. F., BRAUER, A. L., SCHIFFMACHER, A. T. & SETHI, S. 2004. Persistent colonisation by *Haemophilus influenzae* in chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 170**,** 266-272.

MURPHY, T. F., BRAUER, A. L., SETHI, S., KILIAN, M., CAI, X. & LESSE, A. J. 2007. *Haemophilus haemolyticus*: A human respiratory tract commensal to be distinguished from *Haemophilus influenzae*. *Journal of Infectious Diseases,* 195**,** 81-89.

MURPHY, T. F., FADEN, H., BAKALETZ, L. O., KYD, J. M., FORSGREN, A., CAMPOS, J., VIRJI, M. & PELTON, S. I. 2009. Non-typeable *Haemophilus*

influenzae as a pathogen in children. *The Pediatric Infectious Disease Journal,* 28**,** 43-48.

MURPHY, T. F., SETHI, S., KLINGMAN, K. L., BRUEGGEMAN, A. B. & DOERN, G. V. 1999. Simultaneous respiratory tract colonisation by multiple strains of non-typeable *Haemophilus influenzae* in chronic obstructuve pulmonary disease: Implications for antibiotic therapy. *Journal of Infectious Diseases,* 180**,** 404-409.

MURPHY, T. J. & SETHI, S. 1992. Bacterial infection in chronic obstructive pulmonary disease. *American Review of Respiratory Disease,* 146**,** 1067-1083.

MURRAY, A. E., HOLLIBAUGH, J. T. & ORREGO, C. 1996. Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Applied and Environmental Microbiology,* 62**,** 2676-2680.

MURRAY, T. S., EGAN, M. & KAZMIERCZAK, B. I. 2007. *Pseudomonas aeruginosa* chronic colonisation in cystic fibrosis patients. *Current Opinion in Pediatrics,* 19**,** 83-88.

MUYZER, G. 1999. DGGE/TGGE a method for identifying genes from natural ecosystems. *Current Opinion in Microbiology,* 2**,** 317-322.

MUYZER, G., DE WAAL, E. C. & UITTERLINDEN, A. G. 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Applied and Environmental Microbiology,* 59**,** 695-700.

MUYZER, G. & SMALLA, K. 1998. Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie van Leeuwenhoek,* 73**,** 127-141.

MYERS, R. M., FISCHER, S. G., LERMAN, L. S. & MANIATIS, T. 1985. Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Research,* 13**,** 3131-3145.

NAKATSU, C. H. 2007. Soil microbial community analysis using denaturing gradient gel electrophoresis. *Soil Science Society of America Journal,* 71**,** 563-571.

NELSON, A. 2011. *An investigation of the polymicrobial nature of lower respiratory tract infections in cystic fibrosis patients.* Ph. D., University of Northumbria at Newcastle.

NELSON, A., DE-SOYZA, A., BOURKE, S. J., PERRY, J. D. & CUMMINGS, S. P. 2010. Assessment of sample handling practices on microbial activity in sputum samples from patients with cystic fibrosis. *Letters in Applied Microbiology,* 51**,** 272-277.

NICOTRA, M. B., RIVERA, M., DALE, A. M., SHEPHERD, R. & CARTER, R. 1995. Clinical, pathophysiologic, and microbiologic characterisation of bronchiectasis in an aging cohort. *Chest,* 108**,** 955-961.

NISHIOKA, M., MIZUGUCHI, H., FUJIWARA, S., KOMATSUBARA, S., KITABAYASHI, M., UEMURA, H., TAKAGI, M. & IMANAKA, T. 2001. Long and accurate PCR with a mixture of KOD DNA polymerase and its exonuclease deficient mutant enzyme. *Journal of Biotechnology,* 88**,** 141-149.

NOCKER, A., BURR, M. & CAMPER, A. K. 2007. Genotypic microbial community profiling: A critical technical review. *Microbial Ecology,* 54**,** 276-289.

NOLLER, H. F. & NOMURA, M. 1996. Ribsomes. *In:* NEIDHARDT, F. C. (ed.) *Escherichia coli and Salmonella: Cellular and molecular biology.* 2nd ed. Washington, D.C.: ASM Press.

NORRIS, K. A. & MORRIS, A. 2011. Pneumocystis infection and the pathogenesis of chronic obstructive pulmonary disease. *Immunologic Research,* 50**,** 175-180.

NØRSKOV-LAURITSEN, N., OVERBALLE, M. D. & KILIAN, M. 2009. Delineation of the species *Haemophilus influenzae* by phenotype, multilocus sequence phylogeny, and detection of marker genes. *Journal of Bacteriology,* 191**,** 822-831.

NOVAIS, R. C. & THORSTENSON, Y. R. 2011. The evolution of pyrosequencing for microbiology: From genes to genomes. *Journal of Microbiological Methods,* 86**,** 1-7.

NÜBEL, U., ENGELEN, B., FELSKE, A., SNAIDR, J., WIESHUBER, A., AMMAN, R. I., WOLFGANG, L. & BACKHAUS, H. 1996. Sequence heterogeneties of genes encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. *Journal of Bacteriology,* 178**,** 5636-5643.

O'DONNELL, A. E. 2008. Bronchiectasis. *Chest,* 134**,** 815-823.

OLLIS, D. L., BRICK, P., HAMLIN, R., XUONG, N. G. & STEITZ, T. A. 1985. Structure of large fragment of *Escherichia coli* DNA polymerase I complexed with dTMP. *Nature,* 313**,** 762-766.

OLSEN, G. J., LANE, D. J., GIOVANNONI, S. J., PACE, N. R. & STAHL, D. A. 1986. Microbial ecology and evolution: A ribosomal RNA approach. *Annual Review of Microbiology,* 40**,** 337-365.

ORMENO-ORRILLO, E., VINUESA, P., ZUNIGA-DAVILA, D. Z. & MARTINEZ-ROMERO, E. 2006. Molecular diversity of native bradyrhizobia isolated from lima bean (*Phaseolus lunatus L.)* in Peru. *Systematic and Applied Microbiology,* 29**,** 253-262.

PACE, N. R. 2009. Mapping the tree of life: Progress and prospects. *Microbiology and Molecular Biology Reviews,* 73**,** 565-576.

PACE, N. R., SAPP, J. & GOLDENFIELD, N. 2012. Phylogeny and beyond: Scientific, historical, and conceptual significance of the first tree of life. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 1011-1018.

PACE, N. R., STAHL, D. A., LANE, D. J. & OLSEN, G. J. 1986. The analysis of natural microbial-populations by ribosomal-RNA sequences. *Advances in Microbial Ecology,* 9**,** 1-55.

PALLERONI, N. J. 1997. Prokayotic diversity and the importance of culturing. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology,* 72**,** 3-19.

PALMER, C., BIK, E. M., DIGIULIO, D. B., RELMAN, D. A. & BROWN, P. O. 2007. Development of the human infant intestinal microbiota. *PLoS Biology,* 5**,** e177.

PALMER, R. J., JR., KAZMERZAK, K., HANSEN, M. C. & KOLENBRANDER, P. E. 2001. Mutualism versus independence: Strategies of mixed-species oral bioflims *in vitro* using saliva as the sole nutrient source. *Infection and Immunity,* 69**,** 5794-5804.

PAPI, A., BELLETTATTO, M., BRACCIONI, F., ROMAGNOLI, M., CASOLARI, P., CARAMORI, G., FABBRI, L. M. & JOHNSTON, S. L. 2006. Infections and airway inflammation in chronic obstructive pulmonary disease severe exacerbations. *American Journal of Respiratory and Critical Care Medicine,* 173**,** 1114-1121.

PASTEUR, M. C., BILTON, D. & HILL, A. T. 2010. British Thoracic Society guideline for non-CF bronchiectasis. *Thorax. Supplement 1,* 65**,** 1-58.

PASTEUR, M. C., HELLIWELL, S. M., HOUGHTON, S. J., WEBB, S. C., FOWERAKER, J. E., COULDEN, R. A., FLOWER, C. D., BILTON, D. & KEOGAN, M. T. 2001. An investigation into causative factors in patients with bronchiectasis. *American Journal of Respiratory and Critical Care Medicine,* 162**,** 1277-1284.

PATEL, I. S., SEEMUNGAL, T. A. R., WILKS, M., LLOYD-OWEN, S. J., DONALDSON, G. C. & WEDZICHA, J. A. 2002. Relationship between bacterial colonisation and the frequency, character, and severity of COPD exacerbations. *Thorax,* 57**,** 759-764.

PAUWELS, R. A., BUIST, A. S., CALVERLEY, P. M. A., JENKINS, C. R. & HURD, S. S. 2001. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop Summary. *American Journal of Respiratory and Critical Care Medicine,* 163**,** 1256-1276.

PAUWELS, R. A. & RABE, K. F. 2004. Burden and clinical features of chronic obstructive pulmonary disease (COPD). *Lancet,* 364**,** 613-620.

PAVLOV, A. R., PAVLOVA, N. V., KOZYAVKIN, S. A. & SLESAREV, A. I. 2004. Recent developments in the optimisation of thermostable DNA polymerases for efficient applications. *Trends in Biotechnology,* 22**,** 253-260.

PELEG, A. Y., HOGAN, D. A. & MYLONAKIS, E. 2010. Medically important bacterial-fungal interactions. *Nature Reviews Microbiology,* 8**,** 340-349.

PELTOLA, V. T. & MCCULLERS, J. A. 2004. Respiratory viruses predisposing to bacterial infections: Role of neuraminidase. *The Pediatric Infectious Disease Journal,* 23**,** S87-97.

PERLER, F. B., KUMAR, S. & KONG, H. 1996. Thermostable DNA polymerases. *Advances in Protein Chemistry,* 48**,** 377-435.

PERSSON, R., HITTI, J., VERHELST, R., VANEECHOUTTE, M., PERSSON, R., HIRSCHI, R., WEIBEL, M., ROTHEN, M., TEMMERMAN, M., PAUL, K. & ESCHENBACH, D. 2009. The vaginal microflora in relation to gingivitis. *BMC Infectious Diseases,* 9**,** 6.

PETERS, B. M., JABRA-RIZK, M. A., O'MAY, G. A., COSTERTON, J. W. & SHIRTLIFF, M. E. 2012. Polymicrobial interactions: Impact on pathogenesis and human disease. *Clinical Microbiology Reviews,* 25**,** 193-213.

PETROSINO, J. F., HIGHLANDER, S., LUNA, R. A., GIBBS, R. A. & VERSALOVIC, J. 2009. Metagenomic pyrosequencing and microbial identification. *Clinical Chemistry,* 55**,** 856-866.

PHIET, M., CARRERE, J., CIMON, B., CHABASSE, D., DELHAES, L., SYMOENS, F. & BOUCHARA, J. P. 2009. Occurance and relevance of filamentous fungi in respiratory secretions of patients with cystic fibrosis - a review. *Medical Mycology,* 47**,** 387-397.

POON, K. & MACGREGOR, R. B. 1998. Unusual behaviour exhibited by multi-stranded guanine-rich DNA complexes. *Biopolymers,* 45**,** 427-434.

POON, K. & MACGREGOR, R. B. 2000. Formation and structural determinants of multi-stranded guanine-rich DNA complexes. *Biophysical Chemistry,* 84**,** 205-216.

POSTEC, A., URIOS, L., LESONGEUR, F., OLLIVIER, B., QUERELLOU, J. & GODFROY, A. 2005. Continuous enrichment culture and molecular fingerprinting monitoring to investigate the microbial diversity of thermophiles inhabiting deep-sea hydrothermal ecosystems. *Current Microbiology,* 50**,** 138-144.

POTTER, R. T., ROTMAN, F., FERNÁNDEZ, F., MCNEILL, T. M. & CHAMBERLAIN, J. M. 1968. The bacteriology of the lower respiratory tract: Bronchoscopic study of 100 clinical cases. *American Review of Respiratory Disease,* 97**,** 1051-1061.

POXTON, I. 2010. Bacterial communities in the gut and consequences of upsetting them. *Microbiology Today,* 37**,** 232-235.

PRAGMAN, A. A., KIM, H. B., REILLY, C. S., WENDT, C. & ISAACSON, R. E. 2012. The lung microbiome in moderate and severe chronic obstructive pulmonary disease. *PLos One,* 7**,** e47305.

PRISCU, J., ADAMS, E. E., LYONS, W. B., VOYTEK, M. A., MOGK, D. W., BROWN, R. L., MCKAY, C. P., TAKACS, C. D., WELCH, K. A., WOLF, C. F., KIRSHTEIN, J.

D. & AVCI, R. 1999. Geomicrobiology of subglacial ice above Lake Vostok. *Science,* 286**,** 2141-2144.

PURVEDORJ-GAGE, B., COSTERTON, J. W. & STOODLEY, P. 2005. Phenotypic differentiation and seeding dispersal in non-mucoid and mucoid *Pseudomonas aeruginosa* biofilms. *Microbiology,* 151**,** 1569-1576.

PYBUS, V. & ONDERDONK, A. B. 1999. Microbial interactions in the vaginal ecosystem, with emphasis on the pathogenesis of bacterial vaginosis. *Microbes and Infection,* 1**,** 285-292.

QIAGEN. *Principles of pyrosequencing* [Online]. Available: http://www.pyrosequencing.com/DynPage.aspx?id=7454 [Accessed 5th October 2012].

QIN, J., LI, R., RAES, J., ARUMUGAM, M., BURGDORF, K. S., MANICHANH, C., NIELSEN, T., PONS, N., LEVENEZ, F., YAMADA, T. & ET AL 2010. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature,* 464**,** 59-65.

RABE, K. F., HURD, S., ANZUETO, A., BARNES, P. J., BUIST, S. A., CALVERLEY, P., FUKUCHI, Y., JENKINS, C., RODRIGUEZ-ROISIN, R., VAN WHEEL, C. & ZIELENSKI, J. 2007. Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. GOLD Executive Summary. *American Journal of Respiratory and Critical Care Medicine,* 176**,** 532-555.

RAKHIMOVA, E., WIEHLMANN, L., BRAUER, A. L., SETHI, S., MURPHY, T. F. & TÜMMLER, B. 2009. *Pseudomonas aeruginosa* population biology in chronic obstructive pulmonary disease. *Journal of Infectious Diseases,* 200**,** 1928-1935.

RAMAKRISHNAN, V. 2002. Ribosome structure and the mechanism of translation. *Cell,* 108**,** 557-572.

RAPPÉ, M. S. & GIOVANNONI, S. J. 2003. The uncultured microbial majority. *Annual Review of Microbiology,* 57**,** 369-394.

REARDON, S. 2011. A world of chronic disease. *Science,* 333**,** 558-559.

REID, L. 1950. Reduction in bronchial subdivisions in bronchiectasis. *Thorax,* 5**,** 223-247.

REILING, N., HÖLSCHER, C., FEHRENBACH, A., KRÖGER, S., KIRSCHNING, C. J., GOYERT, S. & EHLERS, S. 2002. Toll-like receptor (TLR)2- and TLR4-mediated pathogen recognition in resistance to airborne infection with *Mycobacterium tuberculosis*. *The Journal of Immunology,* 169**,** 3480-3484.

RENOUF, V., CLAISSE, O., MIOT-SERTIER, C. & LONVAUD-FUNEL, A. 2006. Lactic acid bacteria evolution during winemaking: Use of *rpoB* gene as a target for PCR-DGGE analysis. *Food Microbiology,* 23**,** 136-145.

RETTEDAL, E. A., CLAY, S. & BRÖZEL, V. S. 2010. GC-clamp primer batches yield 16S rRNA gene amplicon pools with variable GC clamps, affecting denaturing gradient gel electrophoresis profiles. *FEMS Microbiology Letters,* 312**,** 55-62.

REYES, A., HAYNES, M., HANSON, N., ANGLY, F. E., HEATH, A. C., ROHWER, F. & GORDON, J. I. 2010. Viruses in the faecal microbiota of monozygotic twins and their mothers. *Nature,* 466**,** 334-338.

REYNOLDS, H. Y. 2011. Bronchoalveolar lavage and other methods to define the human respiratory tract milieu in health and disease. *Lung,* 189**,** 87-99.

REYNOLDS, H. Y. & NEWBALL, H. H. 1974. Analysis of proteins and respiratory cells obtained from human lungs by bronchial lavage. *Journal of Laboratory and Clinical Medicine,* 84**,** 559-573.

RICKARD, A. H., GILBERT, P., HIGH, N. J., KOLENBRANDER, P. E. & HANDLEY, P. S. 2003. Bacterial co-aggregation: An integral process in the development of multi-species biofilms. *Trends in Microbiology,* 11**,** 94-100.

RILEY, T. V. & HOFFMAN, D. C. 1986. Interference with *Haemophilus influenzae* growth by other micro-organisms. *FEMS Microbiology Letters,* 33**,** 55-58.

RIORDAN, J. R., ROMMENS, J. M., KEREM, B. S., ALON, N., ROZMAHEL, R., GRZELCZAK, Z., ZIELENSKI, J., LOK, S., PLAVSIC, N., CHOU, J. L. & ET AL 1989. Identification of the cystic fibrosis gene: Cloning and characterisation of complementary DNA. *Science,* 245**,** 1066-1073.

ROBINSON, C. J., BOHANNAN, B. J. M. & YOUNG, V. B. 2010. From structure to function: The ecology of host-associated microbial communities. *Microbiology and Molecular Biology Reviews,* 74**,** 453-476.

ROGERS, G. B., CARROLL, M. P. & BRUCE, K. D. 2009a. Studying bacterial infections through culture-independent approaches. *Journal of Medical Microbiology,* 58**,** 1401-1418.

ROGERS, G. B., CARROLL, M. P., CONNETT, G. J., SERISIEAR, D. J., HOCKEY, P. M., KEHAGIA, V. & JONES, G. R. 2005a. Bacterial community diversity in the CF lung. *Pediatric Pulmonology,* 40**,** 95-97.

ROGERS, G. B., CARROLL, M. P., SERISIER, D. J., HOCKEY, P. M., JONES, G. & BRUCE, K. D. 2004. Characterisation of bacterial community diversity in cystic fibrosis lung infections by use of 16S ribosomal DNA terminal restriction fragment length polymorphism profiling. *Journal of Clinical Microbiology,* 42**,** 5176-5183.

ROGERS, G. B., CARROLL, M. P., SERISIER, D. J., HOCKEY, P. M., JONES, G., KEHAGIA, V., CONNETT, G. J. & BRUCE, K. D. 2006. Use of 16S rRNA gene profiling by terminal restriction fragment length polymorphism analysis to compare bacterial communities in sputum and mouthwash samples from patients with cystic fibrosis. *Journal of Clinical Microbiology,* 44**,** 2601-2604.

ROGERS, G. B., CARROLL, M. P., SERISIER, D. J., HOCKEY, P. M., KEHAGIA, V., JONES, G. R. & BRUCE, K. D. 2005b. Bacterial activity in cystic fibrosis lung infections. *Respiratory Research,* 6**,** 49.

ROGERS, G. B., DANIELS, T. W., TUCK, A., CARROLL, M. P., CONNETT, G. J., DAVID, G. J. P. & BRUCE, K. D. 2009b. Studying bacteria in respiratory specimens by using conventional and molecular microbiological approaches. *BMC Pulmonary Medicine,* 9**,** 14.

ROGERS, G. B., HART, C. A., MASON, J. R., HUGHES, M., WALSHAW, M. J. & BRUCE, K. D. 2003. Bacterial diversity in cases of lung infection in cystic fibrosis patients: 16S ribosomal DNA (rDNA) length heterogeneity PCR and 16S rRNA terminal restriction fragment length polymorphism profiling. *Journal of Clinical Microbiology,* 41**,** 3548-3558.

ROMÁN, F., CANTÓN, R., PÉREZ-VÁZQUEZ, M., BAQUERO, F. & CAMPOS, J. 2004. Dynamics of long-term colonisation of respiratory tract by *Haemophilus influenzae* in cystic fibrosis patients shows a marked increase in hypermutable strains. *Journal of Clinical Microbiology,* 42**,** 1450-1459.

ROMMENS, J. M., IANNUZZI, M. C., KEREM, B. S., DRUMM, G., DEAN, M., ROZMAHEL, R., COLE, J. L., KENNEDY, D., HIDAKA, N. & ET AL 1989. Identification of the cystic fibrosis gene: Chromosome walking and jumping. *Science,* 245**,** 1059-1065.

ROSADO, A. S., DUARTE, G. F., SELDIN, L. & VAN ELSAS, J. D. 1998. Genetic diveristy of *nifH* gene sequences in *Paenibacillus azotofixans* strains and soil samples analysed by denaturing gradient gel electrophoresis of PCR-amplified gene fragments. *Applied and Environmental Microbiology,* 64**,** 2770-2779.

ROSELL, A., MONSÓ, E., SOLER, N., TORRES, F., ANGRILL, J., RIISE, G., ZALACAÍN, R., MORERA, J. & TORRES, A. 2005. Microbiologic determinants of exacerbation in chronic obstructive pulmonary disease. *Archives of Internal Medicine,* 165**,** 891-897.

RUANO, G. & KIDD, K. K. 1992. Modeling of heteroduplex formation during PCR from mixtures of DNA templates. *Genome Research,* 2**,** 112-116.

SAIKI, R. K., SCHARF, S., FALOONA, F., MULLIS, K. B., HORN, G. T., ERLICH, H. A. & ARNHEIM, N. 1985. Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anaemia. *Science,* 230**,** 1350-1354.

SAMUELSON, A., FREIJD, A., JONASSON, J. & LINDBERG, A. A. 1995. Turnover of non-encapsulated *Haemophilus influenzae* in the nasopharynges of otitis-prone children. *Journal of Clinical Microbiology,* 33**,** 2027-2031.

SANDHU, G. S., KLINE, B. C., STOCKMAN, L. & ROBERTS, G. D. 1995. Molecular probes for diagnosis of fungal infections. *Journal of Clinical Microbiology,* 33**,** 2913-2919.

SCHLOSS, P., WESTCOTT, S. L., RYABIN, T., HALL, J. R., HARTMANN, M., HOLLISTER, E. B., LESNIEWSKI, R. A., OAKLEY, B. B., PARKS, D. H., ROBINSON, C. J., SAHL, J. W., STRES, B. & ET AL 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. *Applied and Environmental Microbiology,* 75**,** 7537-7541.

SCHLUENZEN, F., TOCILJ, A., ZARIVACH, R., HARMS, J., GLUEHMANN, M., JANELL, D., BASHAN, A., BARTELS, H., AGMON, I., FRANCESCHI, F. & YONATH, A. 2000. Structure of functionally activated small ribosomal subunit at 3.3 Å resolution. *Cell,* 102**,** 615-623.

SCHUWIRTH, B. S., BOROVINSKAYA, M. A., HAU, C. W., ZHANG, W., VILA-SANJURJO, A., HOLTON, J. M. & CATE, J. H. D. 2005. Structures of the bacterial ribosome at 3.5 Å resolution. *Science,* 310**,** 827-834.

SETHI, S., EVANS, N., GRANT, B. J. B. & MURPHY, T. J. 2002. New strains of bacteria and exacerbations of chronic obstructive pulmonary disease. *New England Journal of Medicine,* 347**,** 465-471.

SETHI, S., MALLIA, P. & JOHNSTON, S. L. 2009. New paradigms in the pathogenesis of chronic obstructive pulmonary disease II. *Proceedings of the American Thoracic Society,* 6**,** 532-534.

SETHI, S. & MURPHY, T. F. 2001. Bacterial infection in chronic obstructive pulmonary disease in 2000: a state of the art review. *Clinical Microbiology Reviews,* 14**,** 336-363.

SETHI, S. & MURPHY, T. F. 2008. Infection in the pathogenesis and course of chronic obstructive pulmonary disease. *The New England Journal of Medicine,* 359**,** 2355-65.

SETHI, S., WRONA, C., ESCHBERGER, K., LOBBINS, P., CAI, X. & MURPHY, T. F. 2007. Inflammatory profile of new bacterial strain exacerbations of chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 177**,** 491-497.

SETHI, S., WRONA, C., GRANT, B. J. B. & MURPHY, T. F. 2004. Strain-specific immune response to *Haemophilus influenzae* in chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 169**,** 448-453.

SHARKEY, D. J., SCALICE, E. R., CHRISTY, K. G., JR., ATWOOD, S. M. & DAISS, J. L. 1994. Antibodies as thermolabile switches: High temperature triggering for the polymerase chain reaction. *Biotechnology,* 12**,** 506-509.

SHEFFIELD, V. C., COX, D. R., LERMAN, L. S. & MYERS, R. M. 1989. Attachment of a 40-base-pair G+C-rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proceedings of the National Academy of Sciences of the United States of America,* 86**,** 232-236.

SIBLEY, C. D., PARKINS, M. D., RABIN, H. R., DUAN, K., NORGAARD, J. C. & SURETTE, M. G. 2008. A polymicrobial perspective of pulmonary infections exposes an enigmatic pathogen in cystic fibrosis patients. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 15070-15075.

SIBLEY, C. D., RABIN, H. & SURETTE, M. G. 2006. Cystic fibrosis: A polymicrobial infectious disease. *Future Microbiology,* 1**,** 53-61.

SLIFKA, M. K., HOMANN, D., TISHON, A., PAGARIGAN, R. & OLDSTONE, M. B. 2003. Measles virus infection results in suppression of both innate and adaptive immune responses to secondary bacterial infection. *The Journal of Clinical Investigation,* 111**,** 805-810.

SOGIN, M. L., MORRISON, H., HUBER, J., WELCH, D. F., HUSE, S., NEAL, P., ARRIETA, J. & HERNDL, G. 2006. Microbial diversity in the deep sea and the underexplored "rare biosphere". *Proceedings of the National Academy of Sciences of the United States of America,* 103**,** 12115-12120.

SOKOL, P. A., LUAN, M. Z., STOREY, D. G. & THIRUKKUMARAN, P. 1994. Genetic rearrangement associated with *in vivo* mucoid conversion of *Pseudomonas aeruginosa* PAO is due to insertion elements. *Journal of Bacteriology,* 176**,** 553-562.

SOLER, N., EWIG, S., TORRES, A., FILELLA, X., GONZALEZ, J. & ZAUBET, A. 1999. Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease. *European Respiratory Journal,* 14**,** 1015-1022.

SOLER, N., TORRES, A., EWIG, S., GONZALEZ, J., CELIS, R., EL-EBIARY, M., HERNANDEZ, C. & RODRIGUEZ-ROISIN, R. 1998. Bronchial microbial patterns in severe exacerbations of chronic obstructive pulmonary disease (COPD) requiring mechanical ventilation. *American Journal of Respiratory and Critical Care Medicine,* 157**,** 1498-1505.

SPENCER, M. D., HAMP, T. J., REID, R. W., FISCHER, L. M., ZEISEL, S. H. & FODOR, A. A. 2011. Association between composition of the human gastrointestinal microbiome and development of fatty liver with choline deficiency. *Gastroenterology,* 140**,** 976-986.

STAINER, R. Y. & VAN NIEL, C. B. 1962. The concept of a bacterium. *Archive für Mikrobiologie,* 42**,** 17-35.

STALEY, J. T. & KONOPKA, A. 1985. Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Annual Review of Microbiology,* 39**,** 321-346.

STANLEY, P. J., WILSON, R., GREENSTONE, M. A., MACWILLIAM, L. & COLE, P. J. 1986. Effect of cigarette smoking on nasal mucociliary clearance and ciliary beat frequency. *Thorax,* 41**,** 519-523.

STEITZ, T. A. 1999. DNA polymerases: Structural diversity and common mechanisms. *The Journal of Biological Chemistry,* 274**,** 17395-17398.

STEITZ, T. A., SMERDON, S. J., JAGER, J. & JOYCE, C. M. 1994. A unified polymerase mechanism for non-homologous DNA and RNA polymerases. *Science,* 266**,** 2022-2025.

STICKLER, D. 1999. Biofilms. *Current Opinion in Microbiology,* 2**,** 270-275.

STOCKLEY, R. A. 1998. Role of bacteria in the pathogenesis and progression of acute and chronic lung infection. *Thorax,* 53**,** 58-62.

STOCKLEY, R. A., MANNINO, D. & BARNES, P. J. 2009. Burden and pathogenesis of chronic obstructive pulomonary disease. *Proceedings of the American Thoracic Society,* 6**,** 524-526.

STRAUSBAUGH, S. D. & DAVIS, P. B. 2007. Cystic fibrosis: A review of epidemiology and pathobiology. *Clinics in Chest Medicine,* 28**,** 279-288.

SUAU, A., BONNET, R., SUTREN, M., GODON, J. J., GIBSON, G. R., COLLINS, M. D. & DORÉ, J. 1999. Direct analysis of genes encoding 16S rRNA from complex microbial communities reveals many more novel molecular species within the human gut. *Applied and Environmental Microbiology,* 65**,** 4799-4807.

SZE, M. A., DIMITRIU, P. A., HAYASHI, S., ELLIOTT, W. M., MCDONOUGH, J. E., GOSSELINK, J. V., COOPER, J., SIN, D. D., MOHN, W. W. & HOGG, J. C. 2012. The lung tissue microbiome in chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 185**,** 1073-1080.

TABETA, K., GEORGEL, P., JANSSEN, E., DU, X., HOEBE, K., CROZAT, K., MUDD, S., SHAMEL, L., SOVATH, S., GOODE, J. & ET AL 2004. Toll-like receptors 9 and 3 as essential components of innate immune defence against mouse cytomegalovirus infection. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 3516-3521.

TAGER, I. & SPEIZER, F. E. 1975. Role of infection in chronic bronchitis. *The New England Journal of Medicine,* 292**,** 563-571.

TAKAGI, M., NISHIOKA, M., KAKIHARA, H., KITABAYASHI, M., INOUE, H., KAWAKAMI, B., OKA, M. & IMANAKA, T. 1997. Characterisation of DNA polymerase from *Pyrococcus* sp. strain KOD1 and its application to PCR. *Applied and Environmental Microbiology,* 63**,** 4504-4510.

TER BRAAK, C. J. F. & ŠMILAUER, P. 2002. *CANOCO Reference Manual and CanoDraw for Windows User's Guide: Software for Canonical Community Ordination (version 4.5),* Ithaca, New York, U.S.A., Microcomputer Power.

THOMAS, D. N. & DIECKMANN, G. S. 2002. Antarctic sea ice — a habitat for extremophiles. *Science,* 295**,** 641-644.

TIZZANO, E. F. & BUCHWALD, M. 1995. CFTR expression and organ damage in cystic fibrosis. *Annals of Internal Medicine,* 123**,** 305-308.

TORVISK, V., DAAE, F. L., SANDAA, R. A. & ØREÄS, L. 1998. Novel techniques for analysing microbial diversity in natural and perturbed environments. *Journal of Biotechnology,* 64**,** 53-62.

TORVISK, V., ØVREÄS, L. & THINGSTAD, T. F. 2002. Prokaryotic diversity — magnitude, dynamics, and controlling factors. *Science,* 296**,** 1064-1066.

TOURLOMOUSIS, P., KEMSLEY, E. K., RIDGWAY, K. P., TOSCANO, M. J., HUMPHREY, T. J. & NARBAD, A. 2010. PCR-denaturing gradient gel electrophoresis of complex microbial communities: A two-step approach to address the effect of gel-to-gel variation and allow valid comparisons across a large dataset. *Microbial Ecology,* 59**,** 776-786.

TSAI, Y. L. & OLSEN, B. H. 1992a. Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. *Applied and Environmental Microbiology,* 58**,** 754-757.

TSAI, Y. L. & OLSEN, B. H. 1992b. Rapid method for separation of bacterial DNA from humic substances in sediments for polymerase chain reaction. *Applied and Environmental Microbiology,* 58**,** 2292-2295.

TULLOCH, D. L., FINCH, L. R., HILLIER, A. J. & DAVIDSON, B. E. 1991. Physical map of the chromosome of *Lactococcus lactis* subsp. *lactis* DL11 and localisation of six putative rRNA operons. *Journal of Bacteriology,* 173**,** 2768-2775.

TUNNEY, M. M., FIELD, T. R., MORIARTY, T. F., PATRICK, S., DOERING, G., MUHLEBACH, M. S., WOLFGANG, M. C., BOUCHER, R. C., GILPIN, D. F., MCDOWELL, A. & ELBORN, J. S. 2008. Detection of anaerobic bacteria in high numbers in sputum from patients with cystic fibrosis. *American Journal of Respiratory and Critical Care Medicine,* 177**,** 995-1001.

TUNNEY, M. M., KLEM, E. R., FODOR, A. A., GILPIN, D. F., MORIARTY, T. F., MCGRATH, S. J., MUHLEBACH, M. S., BOUCHER, R. C., CARDWELL, C., DOERING, G., ELBORN, J. S. & WOLFGANG, M. C. 2011. Use of culture and molecular analysis to determine the effect of antibiotic treatment on microbial community diversity and abundance during exacerbation in patients with cystic fibrosis. *Thorax,* 66**,** 579-584.

TURNBAUGH, P. J., HAMADY, M., YATSUNENKO, T., CANTAREL, B. L., DUNCAN, A., LEY, R. E., SOGIN, M. L., JONES, W. J., ROE, B. A., AFFOURTIT, J. P., EGHOLM, M., HENRISSAT, B., HEATH, A. C., KNIGHT, R. & GORDON, J. I. 2009. A core gut microbiome in obese and lean twins. *Nature,* 547**,** 480-484.

TURNBAUGH, P. J., LEY, R. E., HAMADY, M., FRASER-LIGGETT, C. M., KNIGHT, R. & GORDON, J. I. 2007. The human microbiome project. *Nature,* 449**,** 804-810.

TWISS, J., STEWART, A. W. & BYRNES, C. A. 2006. Longitudinal pulmonary function of childhood bronchiectasis and comparison with cystic fibrosis. *Thorax,* 61**,** 414-418.

VALDIVIA-ARENAS, M. A., AMER, A., HENNING, L. N., WEWERS, M. D. & SCHLESINGER, L. S. 2007. Lung infections and innate host defense. *Drug Discovery Today: Disease Mechanisms,* 4**,** 73-81.

VAN BELKUM, A., RENDERS, N. H. M., SMITH, S., OVERBEEK, S. E. & VERBURGH, H. A. 2000. Comparison of conventional and molecular methods for the detection of bacterial pathogens in sputum samples from cystic fibrosis patients. *FEMS Immunology and Medical Microbiology,* 27**,** 51-57.

VAN DE PEER, Y., NICOLAÏ, S., RIJK, P. D. & WACHTER, R. D. 1996. Database on the structure of small ribosomal subunit RNA. *Nucleic Acids Research,* 24**,** 86-91.

VAN DE WIELE, T., BOON, N., POSSEMIERS, S., JACOBS, H. & VERSTRAETE, W. 2004. Prebiotic effects of chicory inulin in the simulator of the human intestinal microbial ecosystem. *FEMS Microbiology Ecology,* 51**,** 143-153.

VAN DER GAST, C. J., JEFFERSON, B., REID, E., ROBINSON, T., BAILEY, M. J., JUDD, S. J. & THOMPSON, I. P. 2008. Bacterial diversity is determined by volume in membrane bioreactors. *Environmental Microbiology,* 8**,** 1048-1055.

VAN DER GAST, C. J., WALKER, A. W., STRESSMAN, F. A., ROGERS, G. B., SCOTT, P., DANIELS, T. W., CARROLL, M. P., PARKHILL, J. & BRUCE, K. D. 2011. Partitioning core and satellite taxa from within cystic fibrosis lung bacterial communities. *ISME,* 5**,** 780-791.

VASTAG, E., MATTHYS, H., KOHLER, D., GRONBECK, L. & DAIKELER, G. 1985. Mucociliary clearance and airways obstruction in smokers, ex-smokers, and normal subjects who never smoked. *European journal of respiratory diseases. Supplement,* 139**,** 93-100.

VEERAMACHANENI, S. B. & SETHI, S. 2006. Pathogenesis of bacterial exacerbations of COPD. *COPD: Journal of Chronic Obstructive Pulmonary Disease,* 3**,** 109-115.

VERRA, F., ESCUDIER, E., LEBARGY, F., BERNAUDIM, J. F., DE CREMOUX, H. & BIGNON, J. 1995. Ciliary abnormalities in bronchial epithelium of smokers, ex-smokers, and non-smokers. *American Journal of Respiratory and Critical Care Medicine,* 151**,** 630-634.

VITALI, B., PUGLIESE, C., BIAGI, E., CANDELA, M., TURRONI, S., BELLEN, G., DONDERS, G. G. G. & BRIGIDI, P. 2007. Dynamics of vaginal bacterial communities in women developing bacterial vaginosis, candidasis, or no infection, analysed by PCR-denaturing gradient gel electrophoresis and real-time PCR. *Applied and Environmental Microbiology,* 73**,** 5731-5741.

VON WINTZINGERODE, F., GÖBEL, U. B. & STACKEBRANDT, E. 1997. Determination of microbial diversity in environmental samples: Pitfalls of PCR-based rRNA analysis. *FEMS Microbiology Reviews,* 21**,** 213-229.

WAUTERS, G., CHARLIER, J., JANSSENS, M. & DELMEE, M. 2000. Identification of *Arthrobacter oxydans*, *Arthrobacter lutelous* sp. nov. and *Athrobacter albus* sp. nov.

isolated from human clinical specimens. *Journal of Clinical Microbiology,* 38**,** 2412-2415.

WEDZICHA, J. A. 2004. Role of viruses in exacerbations of chronic obstructive pulmonary disease. *Proceedings of the American Thoracic Society,* 1**,** 115-120.

WEDZICHA, J. A. & WILKINSON, T. 2006. Impact of chronic obstructive pulmonary disease exacerbations on patients and payers. *Proceedings of the American Thoracic Society,* 3**,** 218-221.

WEILAND, C. W., KNAPP, S., FLORQUIN, S., DE VOS, A. F., TAKEDA, K., AKIRA, S., GOLENBOCK, D. T., VERBON, A. & VAN DER POLL, T. 2004. Non-mannose-capped lipoarabinomannan induces lung inflammation via Toll-like receptor 2. *American Journal of Respiratory and Critical Care Medicine,* 170**,** 1367-1374.

WEINRICH, U. M. & KORSGAARD, J. 2008. Bacterial colonisation of lower airways in health and chronic lung disease. *The Clinical Respiratory Journal,* 2**,** 116-122.

WELLS, A. U., DESAI, S., WHETTON, C., WILSON, R. & COLE, P. J. 1993. The isolation of *Pseudomonas aeruginosa* from sputum in idiopathic bronchiectasis: An association with extensive disease and severe airflow obstruction. *American Review of Respiratory Disease,* 147**,** A645.

WESTFALL, B., SITARAMAN, K., LEE, J. E., BORMAN, J. & RASHTCHIAN, A. 1999. Platinum *Pfx* DNA polymerase for high-fidelity PCR. *Focus,* 21**,** 46-48.

WEYCKER, D., EDELSBERG, J., OSTER, G. & TINO, G. 2005. Prevalence and economic burden of bronchiectasis. *Clinical Pulmonary Medicine,* 12**,** 205-209.

WHITE, A. J., GOMPERTZ, S. & STOCKLEY, R. A. 2003. Chronic obstructive pulmonary disease: The aetiology of exacerbations of chronic obstructive pulmonary disease. *Thorax,* 58**,** 73-80.

WHITMAN, W. B., COLEMAN, D. C. & WIEBE, W. J. 1998. Prokaryotes: The unseen majority. *Proceedings of the National Academy of Sciences of the United States of America,* 95**,** 6578-6583.

WHITTAKER, R. H. 1969. New concepts of kingdoms of organisms. *Science,* 163**,** 150-160.

WHITTAKER, R. H. 1972. Evolution and measurement of species diversity. *Taxon,* 21**,** 213-251.

WHITWELL, F. 1952. A study of the pathology and pathogenesis of bronchiectasis. *Thorax,* 7**,** 213-219.

WIDMER, F., SCHAFFER, B. T., PORTEOUS, L. A. & SEIDLER, R. J. 1999. Analysis of *nifH* gene pool complexity in soil and litter at a Douglas fir forest site in the Oregon Cascade Mountain Range. *Applied and Environmental Microbiology,* 65**,** 374-380.

WILKINSON, T. M. A., HURST, J. R., PERERA, M. W., DONALDSON, G. C. & WEDZICHA, J. A. 2006. Effect of interactions between lower airway bacterial and rhinoviral infection in exacerbations of COPD. *Chest,* 129**,** 317-324.

WILKINSON, T. M. A., PATEL, I. S., WILKS, M., DONALDSON, G. C. & WEDZICHA, J. A. 2003. Airway bacterial load and FEV1 decline in patients with chronic obstructive pulmonary disease. *American Journal of Respiratory and Critical Care Medicine,* 167**,** 1090-1095.

WILLNER, D., FURLAN, M., HAYNES, M., SCHMIEDER, R., ANGLY, F. E., SILVA, J., TAMMADONI, S., NOSRAT, B., CONRAD, D. & ROHWER, F. 2009. Metagenomic analysis of respiratory tract DNA viral communities in cystic fibrosis and non-cystic fibrosis individuals. *PLoS One,* 4**,** e7370.

WILLNER, D., HAYNES, M. R., FURAN, M., SCHMIEDER, R., LIM, Y. W., RAINEY, P. B., ROHWER, F. & CONRAD, D. 2012. Spatial distribution of microbial communities in the cystic fibrosis lung. *ISME,* 6**,** 471-474.

WILSON, C. B., JOMNES, P. W., O'LEARY, C. J., HANSELL, D. M., COLE, P. J. & WILSON, R. 1997. Effect of sputum bacteriology on the quality of life of patients with bronchiectasis. *European Respiratory Journal,* 10**,** 1754-1760.

WILSON, R. & BOYTON, R. 2006. Bronchiectasis. *In:* LAURENT, G. J. & SHAPIRO, S. D. (eds.) *Encyclopedia of Respiratory Medicine.* Oxford, U.K.: Elsevier Science & Technology Books.

WILSON, R., DOWLING, R. B. & JACKSON, A. D. 1996. The biology of bacterial colonization and invasion of the respiratory tract mucosa. *European Respiratory Journal,* 9**,** 1523-1530.

WIMBERLY, B. T., BRODERSEN, D. E., CLEMONS, W. M., MORGAN-WARREN, R. J., CARTER, A. J., VONRHEIN, C., HARTSCH, T. & RAMAKRISHNAN, V. 2000. The structure of the 30S ribosomal subunit. *Nature,* 407**,** 327-339.

WOESE, C. R. 1987. Bacterial evolution. *Microbiological Reviews,* 51**,** 221-271.

WOESE, C. R. & FOX, G. E. 1977. Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proceedings of the National Academy of Sciences of the United States of America,* 74**,** 5088-5090.

WOESE, C. R., KANDLER, O. & WHEELIS, M. L. 1990. Towards a natural system of organisms: Proposals for the domains Archaea, Bacteria, and Eucarya. *Proceedings of the National Academy of Sciences of the United States of America,* 87**,** 4576-4579.

WOESE, C. R., STACKERBRANDT, E., MACKE, T. J. & FOX, G. E. 1985. A phylogenetic definition of the major eubacterial taxa. *Systematic and Applied Microbiology,* 6**,** 143-151.

WORLD HEALTH ORGANIZATION 2002. The molecular and genetic epidemiology of cystic fibrosis.

YEAGER, C. M., NORTHUP, D. E., GROW, C. C., BARNS, S. M. & KUSKE, C. R. 2005. Changes in nitrogen-fixing and ammonia-oxidising bacterial communities in soil of a mixed conifer forest after wildfire. *Applied and Environmental Microbiology,* 71**,** 2713-2722.

YU, T. W., BAI, L., CLADE, D., HOFFMAN, D., TOELZER, S., TRINH, K. Q., XU, J., MOSS, S. J., LEISTNER, E. & FLOSS, H. G. 2002. The biosynthetic gene cluster of the maytansinoid antitumour agent ansamitocin from *Actinosynnemapretiosum*. *Proceedings of the National Academy of Sciences of the United States of America,* 99**,** 7968-7973.

YU, Z. & MORRISON, M. 2004. Comparisons of different hypervariable regions of *rrs* genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Applied and Environmental Microbiology,* 70**,** 4800-4806.

ZASS, A. K. & SCHWARTZ, D. A. 2005. Innate immunity and the lung: Defense at the interface between the host and environment. *Trends in Cardiovascular Medicine,* 15**,** 195-202.

ZHOU, X., BROWN, C. J., ABDO, Z., DAVIS, C. C., HANSMANN, M. A., JOYCE, P., FOSTER, J. A. & FORNEY, L. J. 2007. Differences in the composition of vaginal microbial communities found in healthy Caucasian and black women. *ISME,* 1**,** 121-133.

ZOETENDAL, E. G., AKKERMANS, A. D. & DE VOS, W. M. 1998. Temperature gradient gel electrophoresis analysis of 16S rRNA from human feacal samples reveals stable and host-specific communities of active bacteria. *Applied and Environmental Microbiology,* 64**,** 3854-3859.

Appendix 1: Chemicals and media

A1.1 1 % ($^{w}/_{v}$) agarose gel

1. 0.3 gm of agarose (*MELFORD*) was added to 30 mL of of 1× TAE buffer solution and boiled until fully dissolved.
2. Five µL of SYBR® Safe DNA gel stain (10 000× concentrate in dimethyl sulphoxide (DMSO)) (Invitrogen™) was then added to the molten agarose, and mixed before being poured into the gel casting chamber.
3. The gel was then left to set in order for the rest of the electrophoresis experiment to continue.

A1.2 Molecular biology dyes

A1.2.1 10 mL 6× bromophenol blue loading dye

1. 0.025 g of bromophenol blue (SIGMA®) was added to 4 g of sucrose (SIGMA®) in an empty 25 mL plastic universal tube.
2. These two chemicals were then made up to 10 mL with dH_2O.

A1.2.2 10 mL DCode™ dye solution reagent

1. 0.05 g of bromophenol blue (SIGMA®) was added to an empty 25 mL plastic universal tube.
2. 0.05 g of xylene cyanol FF (SIGMA®) was then added to the universal tube.
3. The contents of which were then made up to 10 mL with 1× TAE buffer solution.

A1.2.3 10 mL 2× DCode™ gel loading dye

1. 0.25 mL of a 2 % bromophenol blue solution (0.02 g of bromophenol blue (SIGMA®) made up to 1 mL with dH_2O) was added to an empty 25 mL plastic universal tube.
2. 0.25 mL of a 2 % xylene cyanol solution (0.02 g of xylene cyanol FF (SIGMA®) made up to 1 mL with dH_2O) was then added to the universal tube.
3. 7 mL of a 100 % glycerol solution (Fisher Scientific) was then added.
4. This solution was then made up to 10 mL by the addition of 2.5 mL of dH_2O.

A1.3 Denaturing solutions for DGGE experiments

A range of denaturing solutions were utilised for DGGE analysis in a variety of different sample types. The reagent composition and amounts used in each denaturing solution are summarised below:

100 mL denaturing solution (%age denaturant)				
Reagent	**32.5 %**	**35 %**	**40 %**	**60 %**
40 % ($^{v}/_{v}$) 37.5:1 acrylamide:bis-acrylamide (SIGMA® Life Sciences)	30 mL	30 mL	30 mL	30 mL
50× TAE	2 mL	2 mL	2 mL	2 mL
Deionised formamide (SIGMA® Life Sciences)	13 mL	14 mL	16 mL	24 mL
Urea (electrophoresis grade) (MELFORD)	13.65 g	14.7 g	16.8 g	25.5 g
dH_2O	To 100 mL	To 100 mL	To 100 mL	To 100 mL

A1.4 10 % ($^{w}/_{v}$) ammonium persulphate (APS)

1. 1 g of ammonium persulphate (SIGMA®) was weighed out and then decanted into an empty 25 mL plastic universal tube.
2. This was then made to 10 mL using dH_2O, before being aliquoted into 500 µL amounts in 1.5 mL microfuge tubes.
3. These aliquots were then stored at -20 °C until further requirement.

A1.5 Running buffers for gel-based experiments

A1.5.1 2 L 50× TAE solution

1. 200 mL of a 0.5 M EDTA solution pH 8.0 was made up first (37.22 g EDTA made up to 200 mL of dH_2O at pH 8.0 (this must be at pH 8.0 otherwise all of the EDTA will not entirely dissolve into solution)).
2. 200 mL of the EDTA (0.5 M, ph 8.0) solution was then decanted into a 2 L DURAN® glass bottle before 484 gm of Tris-HCl (*MELFORD*) was added.
3. Following this, 114.2 mL of glacial acetic acid (Fisher Scientific) was added.
4. Then, the resulting solution was made up to 2 L using dH_2O.

A1.5.2 1 L 1× TAE solution

1. 20 mL of 50× TAE solution was decanted into a 1 L DURAN® glass bottle.
2. This was then made up to 1 L using dH_2O.

A1.6 Bacteriology and mycology media

A1.6.1 Basic recipe for 1 L Luria-Bertani (LB) media and broth

- 10 g of tryptone (OXOID);
- 5 g of yeast (OXOID);
- 5 g of sodium chloride (NaCl) (SIGMA®);
- 15 g of agar bacteriological (agar no. 1) (OXOID); (omit this step from the recipe when making LB broth);
- Made up to 1 L in an appropriate conical flask using dH_2O;
- Adjust the pH to 7.0 with NaOH;
- Autoclave at 121 °C for 45 min, cool to 50 ° C and then pour 20 mL into each Petri dish.

A1.6.2 NADsens agar and broth

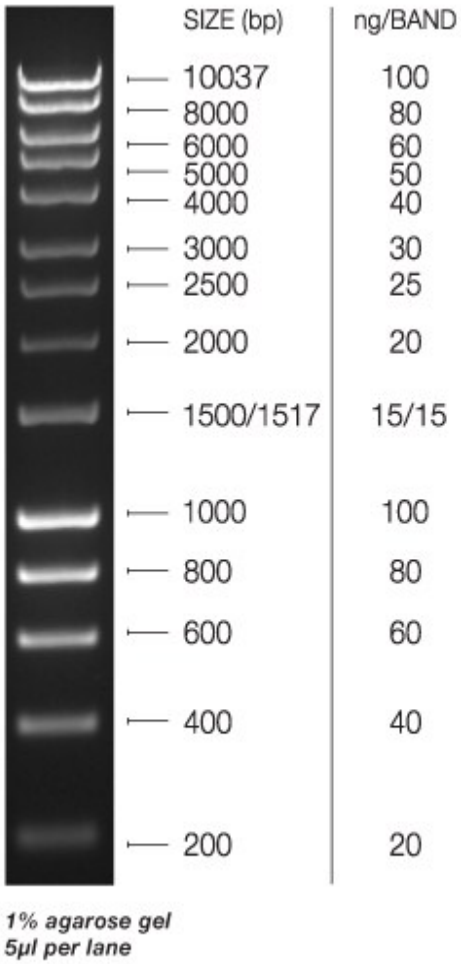
This particular agar and broth was previously prepared at the Freeman Hospital, Microbiology Department, as a requirement of their routine media consortia by an appropriate member of staff. The agar and broth function as an isosensitest, used in antibiotic disk diffusion assays. Briefly, these components are all added together in the following order: hydrolysed casein, peptones, glucose, NaCl, starch, disodium hydrogen phosphate, sodium acetate, magnesium glycerophosphate, calcium gluconate, cobaltous sulphate, cupric sulphate, zinc sulphate, ferrous sulphate, manganous chloride, menadione, cyanocobalamin, L-cysteine hydrochloride, L-typtophan, pyridoxine, pantothenate, nicotinamide, biotin, thiamine, adenine, guanine, xanthine, uracil, and finally agar. These nutrients are all autoclaved at 121 °C for 60 min before cooling down to 50 °C and supplemented with 5 % whole horse blood and 20 mg/L NAD (nicotinamide dinucleotide). In making NADsens broth, as in LB broth, agar is omitted.

A1.6.3 Basic recipe for 100 mL Sabourad broth

1. 100 mL of Sabourad broth was implemented for each fungal strain.
2. 3 g of Sabourad liquid medium (OXOID) was decanted into an appropriate conical flask before being made up to 100 mL with dH_2O.
3. Once Sabourad liquid medium was in solution, this was autoclaved at 120 °C for 45 min.
4. Once sterilised, these broths were now ready for inoculation by the fungal strains already described.

Appendix 2: Molecular base-pair markers

Confirmation of successful PCR experiments and the resulting amplicons were confirmed by comparing the PCR fragments against a known set of molecular base-pair markers and standards shown below (Hyperladder I (BIOLINE)):



Appendix 3: Cross-sectional nCFBR cohort demographic data

Part 1: Phenotypic data

Patient number	Sex	Age	FEV_1 (L)	FEV_1% predicted	Exacerbation present[a]	Recent[b] Abx	Current colomycin	Current azithromycin	Frequent exacerbators[c]
1	F	61	0.67	30	Y	N	N	Y	Y
2	M	67	1.42	46	Y	Y	N	N	Y
3	F	62	1.32	64	Y	Y	N	Y	N
4	M	59	2.02	63.8	N	N	Y	N	Y
5	F	32	3	90	Y	N	N	N	Y
6	F	80	1.35	94	Y	N	N	Y	Y
7	F	63	1.1	49	N	Y	N	Y	Y
8	M	64	2.8	84	N	N	N	Y	N
9	M	58	2.3	70	Y	Y	N	Y	Y
10	M	65	2.25	73	N	N	Y	N	N
11	M	64	1.8	56	N	Y	N	Y	N
12	F	60	1.55	60	N	N	N	N	N
13	F	62	1.9	54	N	N	N	Y	N
14	F	72	0.75	45	Y	N	N	N	N
15	F	68	0.7	53	N	N	N	N	Y
16	M	83	2.2	95	N	N	N	N	N
17	M	68	1.6	52	N	Y	N	N	Y
18	M	65	3.05	101	Y	N	Y	Y	Y
19	F	64	0.5	25	Y	N	N	Y	N
20	F	59	1.2	47	N	Y	Y	Y	Y
21	F	62	2.1	112	Y	N	N	Y	Y
22	F	55	2.5	93	N	N	N	N	Y
23	M	58	0.85	23	N	Y	N	Y	Y
24	F	68	1.65	70	N	N	N	N	N

25	M	35	1.6	39	N	Y	N	N	Y
26	M	75	0.85	27	N	N	N	N	N
27	F	56	0.7	32	N	N	N	N	Y
28	M	58	0.8	24	N	N	N	Y	Y
29	F	63	1.1	54	N	N	N	Y	N
30	F	73	0.9	85	N	N	N	N	N
31	F	62	0.75	33	N	N	Y	Y	N
32	F	72	0.85	37	Y	Y	N	N	Y
33	M	58	1.6	63	N	N	N	Y	N
34	M	29	2.4	56	N	N	N	N	Y
35	F	62	0.6	33	N	N	N	N	N
36	F	79	0.4	27	N	N	N	N	N
37	F	72	0.9	50	N	N	Y	N	N
38	F	52	0.8	34.4	N	N	Y	Y	N
39	F	48	2.5	114	N	N	Y	Y	Y
40	F	71	0.45	25	N	Y	N	Y	N
41	F	67	0.77	39	N	N	N	N	Y
42	F	64	0.59	48	N	N	N	Y	Y
43	M	61	1.27	41	N	N	Y	Y	N
44	F	25	2.45	74	Y	Y	N	Y	Y
45	F	76	1	56	N	N	N	N	Y
46	F	70	0.95	42.3	N	N	N	N	Y
47	F	64	1.05	44.5	N	N	N	N	Y
48	F	72	0.9	50	N	N	N	N	N
49	F	72	0.9	55	Y	Y	N	Y	Y
50	F	71	0.65	41	N	N	N	Y	Y
51	M	74	1	34	Y	N	N	Y	N
52	M	74	0.6	21	Y	Y	Y	Y	Y
53	M	59	2.6	84	N	N	N	N	N
54	F	50	0.8	45	Y	N	N	N	Y
55	F	58	2.4	102	Y	N	N	Y	Y
56	M	60	0.85	28	N	N	N	N	Y

57	F	41	3.2	112	N	N	N	Y	N
58	M	54	2.63	68.7	N	N	N	N	Y
59	M	55	2.4	64	N	N	N	Y	N
60	F	64	1.6	79	Y	N	N	N	N
61	M	18	3.3	69.8	N	N	N	N	N
62	F	33	ND	ND	N	N	N	N	Y
63	F	60	1.55	69	N	N	N	N	Y
64	F	79	0.9	65	N	N	N	N	N
65	M	77	1.9	73	N	N	N	N	Y
66	F	66	2	66	N	Y	N	N	N
67	F	75	0.9	44.6	N	N	N	N	Y
68	F	57	2	99	N	Y	N	N	Y
69	F	66	1.85	96	Y	N	N	N	N
70	F	66	0.55	27	Y	N	N	N	N

[a] As defined by Pasteur, *et al*., (2010) at time of sampling.

[b] Administration within one month of sampling, excluded patient who at the time were being treated with current colomycin and azithromycin therapy.

[c] Defined as those individuals who presented with an exacerbation > 3 times in the last 12 months.

Abbreviations: Abx, antibiotics; FEV_1 (L), forced expiratory volume in one second; FEV_1% predicted, forced expiratory volume in one second %age predicted; ND, not determined from patient's medical notes.

Part 2: Conventional microbiology culture data

Patient number	Cuture-based detection[a]	Culture +ve[b]	PA +ve	HI +ve	Chronic PA infection	Intermittent PA infection	No isolation of PA
1	Mouth flora only	N	N	N	Y	N	N
2	*Haemophilus parainfluenzae*	Y	N	N	N	Y	N
3	Mouth flora only	N	N	N	N	Y	N
4	*Pseudomonas aeruginosa*	Y	Y	N	N	Y	N
5	Mouth flora, *Haemophilus influenzae, Streptococcus pneumoniae*	1	N	Y	N	N	Y
6	Mouth flora only	N	N	N	N	N	Y
7	*Escherichia coli*	Y	N	N	N	Y	N
8	*Candida* spp.	Y	N	N	N	N	Y
9	*Haemophilus parainfluenzae* and *Staphylococcus aureus*	Y	N	N	N	Y	N
10	*Pseudomonas aeruginosa* and *Streptococcus pneumoniae*	Y	Y	N	Y	N	N
11	*Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
12	*Haemophilus influenzae*	Y	N	Y	N	Y	N
13	*Pseudomonas aeruginosa* X 2	Y	Y	N	Y	N	N
14	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
15	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
16	Mouth flora, *Haemophilus parainfluenzae*	Y	N	N	N	N	Y
17	*Pseudomonas aeruginosa* and *Achromobacter xylosoxidans*	Y	Y	N	Y	N	N
18	Mouth flora only	N	N	N	N	N	Y
19	Mouth flora only	N	N	N	N	N	Y
20	Mouth flora only	N	N	N	Y	N	N
21	No growth	N	N	N	N	N	Y
22	Mouth flora only	N	N	N	N	Y	N
23	Mouth flora/ *Stenotrophomonas maltophilia* / MRSA	Y	N	N	Y	N	N
24	*Proteus* spp.	Y	N	N	N	N	Y
25	Mouth flora only	N	N	N	N	Y	N
26	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
27	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
28	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N

29	Mouth flora, *Serratia* spp.	Y	N	N	N	N	Y
30	Mouth flora, *Pseudomonas aeruginosa, Klebsiella pneumoniae*	Y	Y	N	Y	N	N
31	Mouth flora, *Haemophilus influenzae*	Y	N	Y	N	N	Y
32	Mouth flora / *Aspergillus fumigatus*	Y	N	N	N	Y	N
33	Mouth flora, *Haemophilus influenzae*	Y	N	Y	N	Y	N
34	Mouth flora, *Haemophilus influenzae*	Y	N	Y	N	N	Y
35	*Haemophilus influenzae*	Y	N	Y	N	Y	N
36	*Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
37	*Pseudomonas aeruginosa* and *Staphylococcus aureus*	Y	Y	N	Y	N	N
38	P*seudomonas aeruginosa* X 2	Y	Y	N	Y	N	N
39	Mouth flora only	N	N	N	N	N	Y
40	Mouth flora only	N	N	N	Y	N	N
41	*Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
42	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
43	Mouth flora, *Pseudomonas species*	Y	Y	N	Y	N	N
44	Mouth flora, Haemophilus influenzae/Candida spp.	Y	N	Y	N	N	Y
45	*Escherichia coli*	Y	N	N	N	N	Y
46	*Escherichia coli* and *Moraxella catarrhalis*	Y	N	N	N	N	Y
47	*Pseudomonas aeruginosa* X 2	Y	Y	N	Y	N	N
48	Mouth flora only	N	N	N	N	N	Y
49	Mouth flora only	N	N	N	N	Y	N
50	Mouth flora, *Pseudomonas aeruginosa* X 2	Y	Y	N	Y	N	N
51	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
52	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
53	Mouth flora and *Staphylococcus aureus*	Y	N	N	N	Y	N
54	Mouth flora, *Haemophilus influenzae*	Y	N	Y	N	N	Y
55	Mouth flora, *Stenotrophomonas maltophilia*	Y	N	N	N	Y	N
56	Mouth flora only	N	N	N	N	Y	N
57	Mouth flora only	N	N	N	N	Y	N
58	*Haemophilus influenzae*	Y	N	Y	N	N	Y
59	*Morganella morganii*	Y	N	N	N	Y	N
60	Mouth flora, *Haemophilus influenzae*	Y	N	Y	N	N	Y

61	Mouth flora only	N	N	N	N	N	Y
62	Mouth flora, *Pseudomonas aeruginosa*	Y	Y	N	Y	N	N
63	*Pseudomonas aeruginosa*	Y	Y	N	N	N	Y
64	*Haemophilus influenzae*	Y	N	Y	N	N	Y
65	Mouth flora, *Serratia liquefaciens*	Y	N	N	N	N	Y
66	Mouth flora only	N	N	N	N	N	Y
67	Mouth flora only	N	N	N	N	N	Y
68	*Mouth flora and Staphylococcus aureus*	Y	N	N	N	N	Y
69	Mouth flora and *Haemophilus influenzae*	Y	N	Y	N	N	Y
70	Mouth flora only	N	N	N	N	N	Y

[a] Performed by Health & Care Professions Council registered Biomedical Scientists at the Freeman Hospital, Microbiology Department, Newcastle upon Tyne.

[b] The culture of mouth flora in this study was defined as culture-negative.

Abbreviations: HI, *Haemophilus influenzae*; PA, *Pseudomonas aeruginosa*.

Appendix 4: Microbiology Department at the Freeman Hospital SOP: Sputum and related samples

Contained in the following appendix is the official SOP documentation relating to the processing of respiratory tract specimens carried out by the Freeman Hospital; This SOP was generously donated to the author by Prof. John Perry and Audrey Perry. Note that this document has been re-formatted and edited slightly from the original copy to ensure that it meets the requirements of thesis submission.

Newcastle upon Tyne Hospitals NHS Trust:	MIFSOP003
Microbiology Department:	Edition 6.0 26/06/2012
Newcastle upon Tyne Hospitals NHS Trust:	MICRAF009
Microbiology Department:	Edition 3.0 30/01/2012
Location of SOP documentation:	Room 102 FH

Purpose and scope, applications	Sputum samples and other samples such as Pleural fluid, Tissue, NPS BAL and cough swabs may be sent for examination. Samples are inoculated onto culture media and incubated in an appropriate atmosphere and temperature to allow the growth of micro-organisms. General guidance is presented in line with National SOPs. Specific requirements for specimen types are given in checklists.

COSHH, **Health and safety**	Before handling samples the following must be read and understood: COSHH assessments **FH**: F003, F007, F010, F017, F022, F032, F038, F042, F043. The following Risk Assessments must also be read :- **FH** : F001, F002, F012 also those specific to location : F009, R003. All respiratory samples must be processed in a Class 1 safety cabinet wearing appropriate gloves conforming to EN 166. The following documents should also be read before proceeding: Containment level 3 protocols MICH&SF010 Category 3 room fumigation MICH&SF011 Fumigation of Class 1 safety cabinet MICH&SF012

Personnel	All grades of BMS staff and Trainee staff under supervision may perform these procedures.

Introduction

This document describes the isolation of organisms known to cause respiratory infection from associated specimens. Recovery and recognition of organisms responsible for pneumonia depends on

- The adequacy of the lower respiratory tract specimen
- Avoidance of contamination by upper respiratory tract flora
- The use of microscopic techniques and culture methods. If these are suitable, fastidious organisms will grow
- Current and recent antimicrobial treatment

Distinction between tracheobronchial colonisation and true pulmonary infection can prove difficult. Ventilator associated pneumonia carries a high mortality but is difficult to diagnose clinically and microbiologically. The criteria which should be used for diagnosis remain controversial. The poor sensitivity and specificity of sputum culture in the diagnosis of pneumonia in hospitalised ventilated patients has led to the development of a variety of techniques for obtaining lower respiratory tract specimens either with or without the use of fibreoptic bronchoscopy. Specimens which may be received include bronchoalveolar lavage and protected brush specimens collected bronchoscopically, 'blind' protected brush specimens and non directed bronchoalveolar lavage. A pure bacterial count of greater than 10^3 cfu/ml in a brush specimen obtained bronchoscopically has been found to correlate with a histological diagnosis of pneumonia. Brush specimen results and bronchoalveolar lavage results are comparable if a cut off of 10^4 cfu/ml is used for the bronchoalveolar lavage although this is not recommended in this SOP because optional methodology, interpretation and clinical significance remain controversial. Non directed techniques have been found to give results comparable to bronchscopic methods.

Types of specimen

Bronchial aspirate

These are collected by direct aspiration of material from the large airways of the respiratory tract by means of a flexible bronchoscope.

Bronchial brushing

This uses a protected brush catheter in the bronchoscope (a brush within two catheters sealed at the end with a polyethylene glycol plug) to tease material from the airways.

Bronchial washings

Bronchial washings are collected in a similar fashion to bronchial aspirates, but the procedure involves the aspiration of small amounts of instilled saline from the large airways of the respiratory tract.

Pleural fluid

Pleural fluid is the accumulation of fluid between the inner and outer (visceral and parietal) layers of the pleura.

Tracheal aspirate

Tracheal aspirates are collected via the endotracheal tube. They are subject to the same limitations as sputum specimens.

Transthoracic aspirate

These samples are obtained through the chest wall, via a needle passed between the ribs. This procedure may be undertaken to sample, for instance, an aspergilloma, abscess or any focal lung lesion which is accessible. It is rarely used because of the risk of complications.

Transtracheal aspiration

Transtracheal aspiration is also a procedure which carries clinical risks and is therefore rarely performed in the UK. Its clinical usefulness in defining the aetiology of acute bacterial pneumonia has been described. The technique entails the insertion of a large bore needle containing a catheter through the cricothyroid space and into the trachea. The needle is then removed leaving the catheter in place. A syringe attached to the catheter is used to aspirate the secretions. If no material is obtained, 2-3 ml of sterile saline (without antibacterial additives) is injected and aspiration attempted again.

Clinical associations

Empyema

Empyema is the collection of pus in the pleural cavity. It most often occurs as a complication of bacterial infection of the pulmonary parenchyma, either pneumonia or lung abscess.

Any organism can be isolated from pleural fluid, in particular organisms associated with lower respiratory tract infection and organisms acquired by aspiration of the oropharyngeal flora, including oral streptococci and anaerobes. Unusual organisms such as *L. monocytogenes* have been reported.

Organisms particularly associated with empyema in patients with acquired immune deficiency syndrome (AIDS) include:

- *Cryptococcus neoformans*
- *Mycobacterium avium-intracellulare*
- *M. tuberculosis*
- *Nocardia asteroides*

Other organisms which may cause infection in this group of patients include *Pneumocystis carinii* and *Rhodococcus equi.*

Lower respiratory tract infection

Lower respiratory tract infection (LRTI) is a major cause of morbidity and mortality. In the UK, about 50,000 people are admitted to hospital annually, of whom 10% die. The expression LRTI includes pneumonia, where there is inflammation of the lung parenchyma, and infections such as bronchiolitis, which affect the small airways. Lung abscess, where the lung parenchyma is replaced by pus filled cavities, and empyema, where pus occupies the pleural space, are less common manifestations of LRTI.

Pneumonia can be classified according to whether it is community acquired or nosocomial (often defined as being acquired more than 48 hours after hospitalisation). It may be primary, occurring in a person without previously identified risk factors, or secondary. Many conditions are associated with an increased risk of pneumonia. Common risk factors include chronic lung diseases such as chronic obstructive pulmonary disease, diabetes mellitus, cardiac or renal failure and immunosuppression (either congenital or acquired). Reduced level of consciousness and weakness of the gag and cough reflexes are risk factors for aspiration pneumonia. Recent infection with respiratory viruses, particularly influenza, is also a risk factor. There are clinical signs and laboratory indices which can be used to assess the severity of pneumonia in an individual patient, some of which are predictive of an increased risk of death if present.

Pleurisy

Pleurisy is inflammation of the pleura, the serous membranes which cover the lungs and the inner aspect of the thoracic cavity.

Pleural effusion is the accumulation of fluid between the inner and outer (visceral and parietal) layers of the pleura. It may arise as the result of pneumonia or of chronic heart failure or uraemia (when cultures will be negative), or by direct spread of infection, such as a primary tuberculous focus rupturing into the pleural cavity. Carcinomatous involvement of the visceral pleura is one of the more common causes of sterile pleural effusions.

Effusion occurs early in the course of pneumonia representing the pleural response to an inflammatory reaction in the adjacent lung. Bacteria reach the pleural space by various routes:

- spread from an adjacent area of pneumonia
- thoracic surgery or drainage
- bacteraemia
- chest trauma
- trans-diaphragmatic spread from intra-abdominal infection

Tuberculous pleural effusion usually arises as an extension of infection from a sub pleural focus. Only small numbers of bacilli are found in the effusion, as a result microscopy is rarely positive. Pleural biopsy is the specimen of choice.

Pneumonia

The aetiology of pneumonia varies according to whether it has been acquired in the community or in hospital and the risk factors present. Many of the bacteria found as colonisers of the upper respiratory tract have been implicated in pneumonia. Antibiotic treatment and hospitalisation affect the colonising flora, leading to an increase in numbers of aerobic Gram-negative bacilli. These factors affect the sensitivity and specificity of sputum culture as a diagnostic test and results must always be interpreted in the light of the clinical information. Many patients do not have a productive cough and are frequently unable to produce sputum. Sputum culture results are often unreliable and sensitivity of culture is poor for many pathogens.

Community acquired pneumonia

The commonest cause overall is *Streptococcus pneumonia*e, which is responsible for up to 60% of cases in community based surveys. It can affect individuals of any age, including those without known risk factors. Other bacterial pathogens tend to cause pneumonia in the presence of specific risk factors. Patients with COPD are additionally at risk of pneumonia caused by *Haemophilus influenzae* and *Moraxella catarrhali*s. *Staphylococcus aureus* pneumonia occurs either in the context of recent influenza infection or, less commonly, as a result of bloodborne spread from a distant focus, COPD or aspiration. Aerobic Gram-negative rods are rare causes of community acquired pneumonia. Occasionally, *Klebsiella pneumoniae* causes a severe necrotizing pneumonia, typically in patients with a history of alcohol abuse and homelessness (“Friedlanders” pneumonia).

Mycoplasma pneumoniae causes up to 20% of community acquired pneumonia, second only to *Streptococcus pneumoniae.* It tends to occur in epidemics every 4-5 years and affects younger age groups. *Chlamydia pneumoniae* is an exclusively human pathogen, but pneumonia caused by *Chlamydia psittaci* and *Coxiella burnetii* occurs in individuals with the relevant exposure history (birds and farm animals). These agents are responsible for a minority of cases. *Legionella pneumophila* is a rare cause of outbreaks of community acquired pneumonia and about 50% of cases seen in the UK give a recent history of travel. Respiratory viruses, such as RSV, influenza and adenoviruses may occasionally cause primary viral pneumonia. Other rare causes of community acquired pneumonia include *Pasteurella* species and *Neisseria meningitidis.*

Hospital acquired pneumonia

This is the second commonest type of nosocomial infection. Risk is increased by the presence of underlying disease and by various interventions and procedures. Mechanical ventilation is a major risk factor. Aerobic Gram-negative bacilli, including members of the Enterobacteriaceae (such as *Klebsiella* and *Enterobacter* species) and *Pseudomonas aeruginosa* are implicated in up to 60% of cases.

Pneumonia in special situations and patient groups

Aspiration pneumonia:

This occurs when oropharyngeal contents are introduced into the lower respiratory tract. Reduced level of consciousness, for instance following head injury or drug overdose is a risk factor, as are weak gag and cough reflexes which can follow a stroke or other neurological disease. True aspiration pneumonia usually has an insidious onset. Anaerobes such as *Porphyromonas* species, *Fusobacterium, Prevotella* and anaerobic streptococci are implicated and may be part of a mixed infection with aerobic bacteria. Lung abscess formation and empyema are common complications.

Lung abscess:

This may be secondary to aspiration pneumonia, in which case the right middle zone is most frequently affected. Other organisms may give rise to multifocal abscess formation and the presence of multiple small abscesses (<2cm diameter) is sometimes referred to as necrotising pneumonia. Pneumonia caused by *S. aureus* and *K. pneumoniae* may show this picture. Nocardiosis, almost always occurring in a setting of immunosuppression, may present as pulmonary abscesses. Abscesses as a result of blood borne spread of infection from a distant focus may occur in conditions such as infective endocarditis. *Burkholderia pseudomallei* may cause lung abscesses or necrotising pneumonia in those who have visited endemic areas (mainly south East Asia and northern Australia).

Lemierre's syndrome or necrobacillosis originates as an acute oropharyngeal infection. Infective thrombophlebitis of the internal jugular vein leads to septic embolisation and metastatic infection. The lung is most frequently involved and multifocal abscesses may develop. *Fusobacterium necrophorum* is the most common pathogen isolated from blood cultures in patients with this syndrome.

Cystic fibrosis

The major cause of morbidity and mortality in cystic fibrosis patients is progressive pulmonary disease associated with pulmonary infection. The major pathogens are *S. aureu*s, *H. influenza*e, *S. pneumoniae* and pseudomonads, particularly mucoid *P. aeruginosa* strains. Strains of *P. aeruginosa* with differing antibiotic susceptibilities may be isolated from a single sample. Anaerobes may also be present.

Resistance to antibiotics, particularly in *Burkholderia cepacia, Stenotrophomonas maltophilia* (both previously in genus *Pseudomona*s) and *P. aeruginos*a, limits the options for treatment. Transmission of *B. cepacia* between patients may occur and some patients succumb to *"B. cepacia* syndrome" which is a rapidly fulminating pneumonia sometimes accompanied by septicaemia. Nosocomial transmission of *Burkholderia gladioli* has also been reported. Fungi, particularly *Candida* and *Aspergillus* species have also been implicated in infections in cystic fibrosis patients.

Fungal infections

Exposure to environmental fungi is universal and the outcome of exposure is a function of the immune status of the individual and the intrinsic pathogenic potential of the fungus involved. *Candida* species are normal human commensals. *Aspergillus* species and *Pneumocystis carinii* have a worldwide distribution. *Cryptococcus neoformans* is also ubiquitous, but certain serotypes *(C. neoformans var gatti*i) are restricted to tropical and subtropical areas.

Candida species are extremely rare causes of LRTI. Occasionally infection occurs as a result of haematogenous seeding. Diagnosis is difficult given that the airways may become heavily colonised in compromised patients. *Aspergillus fumigatus* is the commonest *Aspergillus* species to infect humans. Pulmonary manifestations in hosts without major impairment of cellular or humoral immunity include allergic bronchopulmonary aspergillosis in patients with asthma or cystic fibrosis and aspergilloma, which occurs when the organism colonises a pre-existing lung cavity or cyst. Invasive pulmonary aspergillosis may have an acute and rapid course in heavily immunosuppressed patients (e.g. allogeneic bone marrow transplant recipients) or a more chronic indolent progression in less compromised patients (e.g. chronic granulomatous disease, HIV infection). Pneumonia caused by *P. carinii* occurs in the setting of impaired cell-mediated immunity such as advanced HIV infection and solid organ or bone marrow transplantation.

Cryptococcal pulmonary infection is unusual in the absence of immune dysfunction and may coexist with extrapulmonary cryptococcosis. Pneumonia may be severe and rapidly progressive in patients with advanced HIV infection but those with better immune function may have a more indolent and variable course. Several other fungi have been reported to cause pulmonary infection in immunocompromised patients, often as part of multisystem disseminated disease. *Penicillium marneffei* infection typically occurs in HIV-infected patients in areas of South East Asia where it is endemic. *Pseudallescheria boydii*, *Fusariu*m, *Alternaria* and zygomycetes have been implicated in infections in patients being treated for haematological malignancy and in bone marrow transplant recipients.

Some unusual fungal causes of LRTI are endemic to defined geographical areas. Although many infections are sub clinical, clinically apparent infections are occasionally imported into the UK. They occur in persons with normal immunity but tend to be more severe in the immunocompromised. The diagnosis should be considered in travellers returning from endemic areas who present with respiratory illness or pneumonia, particularly if they fail to respond to standard therapy. These infections include: histoplasmosis, caused by *Histoplasma capsulatum* (south east USA, Central America); coccidiodomycosis, caused *by Coccidiodes immitis* (south west USA, Central and South America) and blastomycosis caused by *Blastomyces dermatidis* (eastern USA, Africa). Although these infections have distinguishing characteristics, it is often difficult to differentiate them clinically from other causes of respiratory infection, particularly in their early stages. Paracoccidioidomycosis caused by *Paracoccidioides brasiliensis* (Central and South America) usually causes asymptomatic primary pulmonary infection, which may reactivate if immune function declines.

Immunosuppression

Patients with congenital or acquired immune defects are susceptible to infection with unusual organisms which do not cause problems in the normal host. The range of organisms to which an individual is susceptible varies according to the nature of the immune defect. In addition, these patients remain at risk of infection with the common pathogens. Environmental bacteria such as *Nocardia* species, *Rhodococcus equi, Legionella* species and environmental mycobacteria may be implicated. Fungal pathogens such as *Pneumocystis carinii* and *Aspergillus* species are also important as are herpesviruses, particularly cytomegalovirus. Patients with advanced HIV disease and transplant recipients are susceptible to *P. carinii* pneumonia and mycobacterial infection. CMV infection often manifests itself as pneumonitis in heart, lung and heart/lung transplant recipients (but HIV infected individuals only rarely get CMV pulmonary disease). Invasive pulmonary aspergillosis is seen especially in patients who have had prolonged periods of granulocytopenia.

Mycobacterial disease

Primary pulmonary infection with *Mycobacterium tuberculosis* may lead to the formation of the 'primary complex', particularly in childhood. The pulmonary focus may be relatively small, but the draining hilar lymph nodes become greatly enlarged and may rupture, spreading infectious material into other areas of the lung. It is at this stage that miliary spread to other organs may occur via blood and lymphatics. Adolescents and adults may have asymptomatic primary infection, a typical primary complex or infection which progresses to typical chronic cavitating tuberculosis. Chronic cavitating disease is usually seen in reactivated primary infection and the lung apices are most commonly involved. The cough which accompanies this process produces aerosols of infectious particles which are the route by which other persons may become infected.

Nocardia and Actinomyces infections

Nocardiosis and actinomycosis are rare conditions which may affect other systems apart from the lungs. *Nocardia* is most often seen in the lung where it may cause acute, often necrotising, pneumonia. This is commonly associated with cavitation. It may produce a slowly enlarging pulmonary nodule and pneumonia which is often associated with empyema. Immune defects ranging from alcoholism to organ transplantation and HIV infection are present in the majority (60% plus) of patients presenting with nocardiosis. *Actinomyces* species cause a thoracic infection which may involve the lungs, pleura, mediastinum or chest wall. Cases often go unrecognised until empyema or a chest wall fistula develops. Aspiration of oral contents is a risk factor for the development of thoracic actinomycosis, thus predisposing conditions include alcoholism, cerebral infarction, drug overdose, general anaesthesia, seizure,

diabetic coma or shock. The appropriate specimens for investigation of both these organisms are pus, tissue and biopsy specimens.

Parasitic infections

Several helminth infections may give rise to a syndrome characterised by patchy pulmonary infiltrates and eosinophilia accompanied by symptoms of cough, fever and weight loss. These signs and symptoms are associated with passage of larval forms through the lungs and include *Ascaris lumbricoide*s, hookworms and *Strongyloides stercorali*s. The lung fluke, *Paragonimus westermanii* has a wide distribution and is particularly prevalent in the far East, Indian subcontinent and West Africa. Human infection is acquired by consumption of uncooked freshwater crabs or crayfish, which harbour encysted metacercariae. Although infection may be asymptomatic, heavy infestations are manifested by pulmonary infiltrates as above which may progress to chronic productive cough with pleuritic chest pain. Ova of *P. westermanii* are demonstrable in sputum. Refer to parasite SOP for processing of samples for parasite examination.

Specimen processing

SAMPLES SHOULD BE GRADED AS FOLLOWS:

100% pus - Purulent

The sample is stuck to the container and does not move when it is inverted.

75% pus / 25% saliva - Mucopurulent

50% pus / 50% saliva - Mucopurulent

25% pus / 75% saliva - Mucopurulent

100% saliva - Mucoid

The sample may be discarded as unsuitable using the Apex code MUCOID, unless it is from an ICU/HDU, cystic fibrosis or immunocompromised patient.

Preparation of Sputasol

Carefully using forceps peel the metal foil from a fresh vial of Sputasol (Refrigerator Category 3 suite) and discard the foil into a Sharps box.

Using a medical flat containing 100 ml of sterile de-ionised water pour in the vial of Sputasol and mix until dissolved. Using a sterile pipette place the contents of the vial into the remainder of the de-ionised water, tighten cap, mix and label with the current date. Sputasol must not be used if more than 48 hours old.

Store in the Blood Culture refrigerator. Discard the Sputasol glass vial into the Sharps box.

Sputum Culture Protocol

Before culture is commenced check the sample details match those on the request form and proceed to number the appropriate plates according to the tables at the back of the SOP, or the quick guide at the front of the SOP (MIFSOP 033).

Carry the plates and samples into the Category 3 suite in carrier racks and place into the safety cabinet. Switch on the safety cabinet and ensure the air-flow readings are within range before commencing. If not inform a Senior member of staff – **DO NOT PROCEED UNTIL RECTIFIED.**

Put on relevant P.P.E (Disposable gloves and safety gown)

Using a sterile pipette, add an equal volume of Sputasol to the samples, discard the pipette to waste. Tighten the lids securely and vortex until the sample is fully homogenised.

Using a 10 µl loop place a level loopful of sample into 5 ml of sterile de-ionised water, and vortex to homogenise. Discard loop to waste jar.

Using a separate sterile 10 µl loop, inoculate the appropriate plates and spread for single colonies. Discard the loop into the discard jar.

Incubate the plates in appropriate atmospheric conditions for 24 hours, and examine for typical bacterial pathogens as contained in the tables. Re-incubate as required according to the tables.

Store samples in the cold room when finished.

Wipe down the safety cabinet with Terminex after culturing a batch of samples, discard gloves into clinical waste bags and remove gown.

Pleural Fluids Culture Protocol

Upon arrival in the laboratory check that the sample details match those on the request form. In the category 3 suite, place the sample into the sealed centrifuge buckets and balance ready for centrifugation. Spin the sample for 10 minutes at 3000 r.p.m. Turn on the safety cabinet and check readings are acceptable before proceeding.

Pour off the sample supernatant into a labelled sterile universal. Number up the plates as per protocol and place 5 drops of spun deposit onto a vial of sterile saponin (Blood Culture Refrigerator) using a sterile Pasteur pipette. Vortex to mix. Using a 10 µl loop apply 10 µl to the culture plates and spread for single colonies. Discard loop and pipette into discard waste. Incubate the plates under the appropriate atmospheric conditions overnight before reading. See tables for list of appropriate pathogens. Re-incubate as required.

Tissues and Biopsies Culture Protocol

Upon arrival in the laboratory check that the sample details match those on the request form. Label the plates as appropriate and transfer to the safety cabinet. Pour Terminex into autoclavable container and place into the cabinet. Switch on the safety cabinet and ensure the air-flow readings are within range before commencing. If not inform a Senior member of staff – **DO NOT PROCEED UNTIL RECTIFIED.**

Put on relevant P.P.E (Disposable gloves and safety gown)

Place the sample into a tissue grinder, add 3 drops of sterile de-ionised water, and grind the sample ideally until a smooth homogenous suspension is achieved. Occasionally the sample may be difficult to grind, in such cases grind the sample for at least 10 minutes. Using a 10 µl loop place a loopful of the ground tissue suspension onto each plate and spread for single colonies. Discard loop into discard waste. Upon completion of all cultures and enrichments place the grinder into the disinfectant and using a pipette ensure the grinder is filled. Leave to stand overnight before autoclaving.

Incubate the plates under the appropriate atmospheric conditions overnight before reading. See tables for list of appropriate pathogens. Re-incubate as required.

Respiratory Swab Culture Protocol

Occasionally swabs of respiratory samples are sent. These are often from CF patients, lung Tx donors or from anastomosis sites. These are cultured according to the quick guide. The sample is checked to ensure the details match those on the request form. Number the plates as appropriate and inoculate the swab onto the plates and spread for single colonies. Place swab into appropriate rack in cold room upon completion. Incubate the plates under the appropriate atmospheric conditions overnight before reading. See tables for list of appropriate pathogens.

BAL Samples

Introduction

It is possible to recover bacteria, viruses, protozoa and fungi responsible for pulmonary infection from BAL specimens. Although isolation of *Aspergillus* species from BAL is of value in patients with invasive disease, it is only 30% sensitive. BAL specimens are particularly useful in the diagnosis of *Pneumocystis carinii* pneumonia, pneumonia caused by *Legionella pneumophila* and for the detection of *Mycobacterium tuberculosis* presenting as pneumonia.

Bronchoalveolar lavage (BAL)

A segment of lung is `washed' with sterile saline after insertion of a flexible bronchoscope, thus allowing recovery of both cellular and non-cellular components of the epithelial surface of the lower respiratory tract. It is a reliable method for making a definitive aetiological diagnosis of pneumonia and other pulmonary infections.

Non directed bronchoalveolar lavage (NBL)

A suction catheter, preferably a protected BAL catheter to minimise contamination, is passed down the endotracheal tube until resistance is met. An aliquot of sterile saline is injected and then aspirated. This method provides a lower respiratory tract sample without the need for bronchoscopy and without the attendant risks of transtracheal aspiration.

Culture of BAL samples

Before culture is commenced check the sample details match those on the request form and proceed to number the appropriate plates according to the tables at the back of the SOP, or the quick guide at the front of the SOP (MIFSOP 030).

Carry the plates and samples into the Category 3 suite in carrier racks and place into the safety cabinet. Switch on the safety cabinet and ensure the air-flow readings are within range before commencing. If not inform a Senior member of staff – **DO NOT PROCEED UNTIL RECTIFIED.**

Put on relevant P.P.E (Disposable gloves and safety gown).

Place the sample into the sealed centrifuge buckets and balance ready for centrifugation. Spin the sample for 10 minutes at 3000 r.p.m. In the safety cabinet transfer the supernatant into a labelled plastic universal. Add 20 ml of sterile de-ionised water to the deposit and vortex. Centrifuge the sample again and discard the supernatant to another labelled plastic universal. Vortex. The sample is now ready for culture.

Lavages from patients are cultured as neat onto the appropriate plates by placing 10 µl of sample onto the plates and spreading for single colonies.

Lavages from patients with cystic fibrosis and bronchiectasis may also be diluted by placing a 10 µl loopful into 5 ml of sterile de-ionised water and inoculating this diluted sample onto a chocolate plate only. Discard loops into discard waste.

Incubate the plates under the appropriate atmospheric conditions overnight before reading. See tables for list of appropriate pathogens. Re-incubate as required.

Interpretation guidelines

This applies to all routine samples from FRH, RVI, Clinics, Outpatients and GP's. It excludes samples from both Critical Care sites, CF patients and bronchiectasis.

Sensitivities are performed on *Pseudomonas* spp, Coliforms and *S. aureus* when:

- Heavy and pure from a purulent sample
- Or accompanied by good clinical details

Sensitivities are suppressed. The medical staff may add a further comment if necessary.

Charts

BAL Samples (Diagnostic/Donor/Recipient)

Clinical details/ Conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp (°C)	Atmos	Time		
Culture neat only	Blood agar	35-37	5-10% CO_2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* Other organisms in pure growth may be significant.
	CBAC	35-37	5-10% CO_2	24-48 h	Daily	*Haemophilus sp* *Enterobacteriaceae* *Pseudomonads sp.* *Capnocytophagia*
	Legionella media	35-37	CO_2	10 days	Daily	*Legionella sp. Nocardia*
	Gram stain					*Any organisms and cellular examination*
	Send sample to HPA for TB culture					*Mycobacteria*
	Sab	35-37	Air	5 Days	Daily	*Candida sp.* *Aspergillus sp.* Other fungi.
If the patient has cystic fibrosis then add the following:						
	B. cepacia media	35-37	Air	5 days 10-day terminal read	Daily	*B. cepacia, B. gladioli* Atypical *Mycobacteria*
	SAID	35-37	Air	24-48 h	Daily	*S. aureus*

Sputum

General guidance on Culture media, conditions and organisms						
Clinical details/conditions	**Standard media**	**Incubation**			**Cultures read**	**Target organism(s)**
		Temp (°C)	**Atmos**	**Time**		
Routine sputum samples, NB, dilute 10 µl of sample into 5 ml water NP and ET secretions	Blood agar	35-37	5-10% CO_2	24-48 h **Routine GP samples 24 h**	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* Other organisms in pure growth may be significant.
	CBAC	35-37	5-10% CO_2	24-48 h	Daily	*Haemophilus sp* *Enterobacteriaceae* *Pseudomonads sp.* *Capnocytophagia*

Culture all samples even if mucoid from all FH, RVI Critical Care and CF patients.

Bronchiectasis sputum

Clinical details/conditions	Supplementary media	Incubation			Cultures read	Target organism(s)
		Temp (°C)	Atmos	Time		
Dilution	Chocolate agar	35-37	CO_2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* *Pseudomonas sp*
Neat culture	Blood agar	35-37	CO_2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* Other organisms in pure growth may be significant.
	CBAC	35-37	CO_2	24-48 h	Daily	*Haemophilus sp* *Enterobacteriaceae* *Pseudomonads sp.* *Capnocytophagia*

Cystic fibrosis sputum

Clinical details/conditions	Supplementary media	Incubation			Cultures read	Target organism(s)
		Temp (°C)	Atmos	Time		
Dilution	Chocolate agar	35-37	CO_2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* *Pseudomonas sp*
Neat culture	Blood agar	35-37	CO_2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* Other organisms in pure growth may be significant.
	CBAC	35-37	CO_2	24-48 h	Daily	*Haemophilus sp* *Enterobacteriaceae* *Pseudomonads sp.* *Capnocytophagia*
	CLED	35-37	Air	24-48 h	Daily	*Enterobacteriaceae* *Pseudomonas sp*
	Sab	35-37	Air	5 days	Daily	*Aspergillus sp.* And other *fungi* *Candida sp.*
	SAID	35-37	Air	24-48 h	Daily	*S. aureus*
	B. Cepacia media	30	Air	5 Days	Daily 10-day Terminal Read	*B. cepacia, B. gladioli* Atypical *Mycobacteria*

Cough swabs

Clinical details/conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp (°C)	Atmos	Time		
Cough swab Not cystic fibrosis	Blood agar	35-37	5-10% CO_2	24- 48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* Other organisms in pure growth may be significant
	CBAC	35-37	CO2	24-48 h	Daily	*Haemophilus sp* *Enterobacteriaceae* *Capnocytophagia* *Pseudomonas sp.*
Cystic fibrosis	Cepacia media	30	Air	5 days 10 day terminal read	Daily	*B. cepacia* *B .gladioli*

Pleural fluids

Clinical details/Conditions	Supplementary media	Incubation			Cultures read	Target organism(s)
		Temp (°C)	Atmos	Time		
Centrifuge Saponise	Chocolate agar Blood agar	35-37	CO_2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* *Pseudomonas sp*
	Blood agar	35-37	An	24-48 h	Daily	Anaerobes
On request only	Legionella plate	35-37	CO_2	10 days	Daily	*Legionella sp.*
All samples from Freeman Wards 23 and 28	Pneumocoocal antigen testing					Forward sample to serology for testing.

*** TB Sent only on request**

Lung tissue samples, biopsy and PM materials, empyema

Clinical details/Conditions	Supplementary media	Incubation			Cultures read	Target organism(s)
		Temp (°C)	Atmos	Time		
Grind sample in tissue grinder as appropriate	Choc	35-37	CO_2	24-48 h	Daily	*Haemophilus sp* *Enterobacteriaceae* *Pseudomonads sp.* *Capnocytophagia*
	Blood agar	35-37	CO_2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* Other organisms in pure growth may be significant.
	Sab	35-37	Air	5 Days	Daily	*Candida sp.* *Aspergillus sp.* and other fungi.
	Blood Agar and FAA Neo + Mz 5 discs	35-37	An	5 Days	After 48 hours in cabinet then at 5 days	Anaerobes
	Enrichment if required Robertsons Cooked Meat broth	35-37	Air	48 hours	Subculture at 48 hours	Anaerobes. Any other pathogenic sp.
	Legionella plate Not neonates	35-37	CO_2	10 days	Daily	*Legionella sp.*
	TB culture Not neonates					On Empyema samples on Medical Request only
Empyema						**Send to Serology for Pn Antigen**

Lung abscess and aspiration pneumonia sputum

Clinical details/conditions	Standard media	Incubation			Cultures read	Target organism(s)
		Temp (^oC)	Atmos	Time		
Culture Sample Neat	Blood agar	35-37	5-10% CO_2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* Other organisms in pure growth may be significant
	CBAC	35-37	5-10% CO_2	24-48 h	Daily	*Haemophilus sp* *Enterobacteriaceae* *Pseudomonads sp.* *Capnocytophagia*
Not required for aspiration pneumonia	Sab	35-37	Air	5 Days	Daily	*Candida sp.* *Aspergillus sp.* and other fungi.
	Blood Agar and FAA Neo + Mz 5 discs.	35-37	An	5 Days	After 48 hours in cabinet then at 5 days.	Anaerobes

Transplant assessment and post lung transplant sputum

Clinical details/Conditions	Supplementary media	Incubation			Cultures read	Target organism(s)
		Temp (°C)	Atmos	Time		
Culture Neat	CBAC	35-37	CO_2	24-48 h	Daily	*Haemophilus sp* *Enterobacteriaceae* *Pseudomonads sp.* *Capnocytophagia*
	Blood agar	35-37	CO2	24-48 h	Daily	*S. pneumoniae* *M. catarrhalis* *S. aureus* Other organisms in pure growth may be significant
	CLED	35-37	Air	24-48 h	Daily	***Use on CF only.***
	Sab agar	35-37	Air	5 Days	Daily	*Aspergillus sp* and other fungi
Culture dilute onto	Chocolate agar	35-37	CO2	24-48 h	Daily	*H. influenzae, S. pneumoniae*
If CF patient add in the following:	B. cepacia Medium	35-37	Air	5 days 10-Day Terminal read	Daily	*B. cepacia, B. gladioli* Atypical Mycobacteria
	SAID	35-37	Air	24-48	Daily	*S.aureus*
If cystic fibrosis Tx Assessment	TB culture					Forward to HPA Newcastle
If cystic fibrosis TX assessment	B. cepacia broth	35-37	Air	Plate after 48 hrs onto B. cep. media	Read after 72 hrs	*B. cepacia*

Respiratory Sample Quick Guide

CODE	SPECIMEN	BA	CBAC	CHOC	CLED	2D SAB	5D SAB	AN BA	SAID	CEP	LEG Full plate	5D AN (FAA/BA)	ROB	STB	H.D.A	COMMENTS
R1	Sputum:- Routine	Dil	Dil													
R2	Sputum:- Routine Critical Care All CC, ITU, HDU and PICU	Y	Y												Y	Use 1/4 HDA plate
	SCIDS/Immunosuppressed Sputum/ETS/NPS etc															
R5	RVI:- GNCH03, 04, 14, 35SCBU	Y	Y			Y										Use 1/4 SAB plate
R12	Sputum, Bronchiectasis	Y	Y	Dil												
R4	Sputum, CF	Y	Y	Dil	Y		Y		Y	Y					CC	Use 1/4 SCID plate
R12	Sputum, Post LTx	Y	Y	Dil											Y	
R13	Sputum, Post LTx, CF	Y	Y	Dil	Y				Y	Y					Y	
R11	Sputum, Tx Assessment	Y	Y	Dil			Y								Y	
R4A	Sputum, Tx Assessment, CF	Y	Y	Dil	Y		Y		Y	Y				Y	Y	**Add B.cepacia broth**

R9	Sputum, asp.pneumonia/lung abscess	Y	Y			Y					Y				CC		
R2	ETS/NPS	Y	Y												CC		
R2	Cough Swab	Y	Y														Includes bronchiectasis
R6	CF Cough swabs	Y	Y							Y							Use 1/4 CEP plate for C/S
R7	Pleural Fluid	Y		Y			Y								CC		< 18-John P.
R10	Pleural Fluid, Empyema	Y		Y		Y	Y				Y	Y		Y	CC		All get Pn Ag < 18-John P.
R14	BAL*	Y	Y			Y					Y			Y	CC		Gram: Donor/Recip Tx**
R17	BAL*, CF	Y	Y		Y	Y		Y	Y		Y			Y	CC		Gram: Donor/Recip Tx**
R8	Lung biopsy/tissue	Y		Y		Y					Y	Y	Y	Y	CC		10 day AN (Nali/BA)
R18	Neonatal death tissue	Y		Y			Y					Y					
R16	Anastomosis specimens/wash	Y	Y			Y									CC		
R2	Airways/bronchus swab	Y	Y												CC		

* BALS from the same lung can be pooled, but BALs from different lungs must be processed separately.

** Label hospital number as GDON + hospital No. and names as DONOR + surname of recipient. Inform Prof. Gould/Crit Care cover with Gram-stain result direct sens may be required.

Appendix 5: COPD cohort demographic data

Phenotypic and conventional microbiology culture data

Sample number	Sex	Age	Height (m)	FEV_1% predicted	GOLD stage	Ex-smoker	Current smoker	BAL microbiology data[a]
CS#1	M	71	1.58	43	III	Y	N	*Streptococcus pneumoniae*
CS#3	M	67	1.61	49	III	Y	N	*Candida* spp., & *Haemophilus influenzae*
CS#4	M	52	1.64	68	II	Y	N	No pathogens isolated
CS#5	M	63	1.73	39	III	N	Y	No pathogens isolated
CS#6	M	64	1.83	49	III	Y	N	*Chryseobacterium indologenes*
CS#7	M	63	1.83	60	II	Y	N	No pathogens isolated
CS#8	M	69	1.67	43	III	N	Y	*Moraxella catarrhalis* & *Haemophilus influenzae*
CS#9	F	73	1.22	65	II	Y	N	No pathogens isolated
CS#10	M	61	1.74	43	III	N	Y	*Moraxella catarrhalis*
CS#11	M	62	1.73	36	III	Y	N	No pathogens isolated
CS#12	F	70	1.61	57	II	Y	N	No pathogens isolated

[a] CS#11 culture data was based on a bronchial secretion sample as patient was unable to produce BAL sample.

Abbreviations: BAL, bronchoalveolar lavage; CS#X, COPD subject number; FEV_1% predicted, forced expiratory volume in one second percentage predicted; GOLD, Global Initiative for Chronic Obstructive Lung Disease.